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(54) Title: ANAEROBE TARGETED ENZYME-MEDIATED PRODRUG THERAPY

(57) Abstract: Recombinant anaerobic bacterial compositions that under anaerobic conditions present in a solid tumor and produce an enzyme capable of catalyzing the conversion of a prodrug to its cytotoxic product in situ are described. Methods of treating tumors using the composition are also described.

ANAEROBE TARGETED ENZYME-MEDIATED PRODRUG THERAPY

Field of the Invention

This invention relates to genetically-engineered bacteria which, under
5   anaerobic conditions present in a solid tumor and produce an enzyme capable of
catalyzing the conversion of a prodrug to its cytotoxic product *in situ* and methods
of treating tumors using the same.

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Background of the Invention

Despite the success of modern chemotherapy in the treatment of certain
5   types of leukemia and lymphoma and some other relatively rare cancers, few
current anticancer therapies have met with significant clinical success in treatment
of the most common forms of cancer. The poorly responsive cancers are typically
solid tumors comprising both proliferating and non-proliferating cells. Typically, the
anticancer drugs used today are effective predominantly against rapidly
10   proliferating tumor cells, and are toxic to rapidly proliferating non-cancerous cells.
Two general approaches are currently being pursued to overcome this problem.
First, there is an intensive search for new drugs that are selective for cancer cells.
A second strategy is that of targeting new or existing drugs specifically to tumors.
Many attempts at applying this strategy have relied on monoclonal antibodies
15   (Mabs) to carry a drug or toxin to the tumor. A major problem with this approach is
tumor cell heterogeneity and the inability to deliver antibody conjugates to every
tumor cell (Jain, 1989). One way of overcoming this problem is the use of
enzyme-antibody conjugates which activate prodrugs to form diffusible cytotoxins
(Bagshawe, 1989). This approach has been called antibody-directed enzyme
20   prodrug therapy (ADEPT). The two-step ADEPT process requires the conjugation
of a suitable enzyme to a monoclonal antibody which localizes the enzyme to the
tumor. When most of the nonbound antibody-enzyme conjugate has been
cleared, a prodrug is administered which can be activated by the enzyme to a
cytotoxic species (Bagshawe, 1990). Though the ADEPT strategy is promising, it
25   has a number of problems. First, the large majority of the Mab-enzyme conjugates
do not localize in the tumor, and studies have shown that concentrations of the
active drug in normal tissues can be greater than in the tumor (Antonio *et al.*,
1990). Also, MAbs of high enough specificity are not available for many tumors.
See, also Melton *et al.*, 1996.
30   Other targeting approaches include the use of recombinant toxins, such as
growth factors fused to a bacterial toxin (Fitzgerald *et al.*, 1992), and the use of
tumor-infiltrating lymphocytes genetically engineered to produce a protein such as
tumor necrosis factor (TNF) (Rosenberg, 1992). No reports of improved activity of
any of these targeting strategies has yet appeared.

4

In still another approach, a gene for the cancerostatic polypeptide Colicin E3 was introduced into an uncharacterized mixture of endogenous plasmids in *C. oncolyticum (C. butyricum* M-55, renamed because of its oncolytic activity; Schlechte, *et al.*, 1988). This approach, however, was unsuccessful based on the

5   inability to transform *C. oncolyticum* and the corresponding inability to show that the recombinants were expressing active protein. In addition, this approach is based on an anticancer agent that is a protein rather than a low molecular weight compound. At the present time, low molecular weight compounds constitute the majority of chemotherapeutic agents.

10   Human solid tumors, which make up more than 90% of all human cancers are known to comprise a hypoxic environment in that they are considerably less well oxygenated than normal tissues. Recent cancer treatment strategies focused on this aspect include the use of drugs that are toxic only under hypoxic conditions, *e.g.*, tirapazamine, and gene therapy approaches based on the use of

15   transcription factors that are inducible under hypoxic conditions, *e.g.*, hypoxia-inducible factor 1 (HIF-1). See, *e.g.*, Brown, 2000.

Despite advances in cancer treatment, significant side effects due to the toxicity of the chemotherapeutic agents in current use remains a problem. Gene therapy strategies have been attempted and are the subject of ongoing clinical

20   trials. However, the lack of specificity of delivery systems and toxic side effects due to those delivery systems must be overcome in order for such strategies to have clinical relevance.

Accordingly, a need exists for selective targeting of toxic chemotherapeutic agents to solid tumor tissue without exposing healthy tissue to the agent.

25

Summary of the Invention

The present invention relates to transformation of obligately anaerobic bacteria with an recombinant expression vector comprising a coding sequence for an enzyme capable of catalyzing the conversion of a prodrug into a cytotoxic agent

30   *in vivo.*

In one embodiment, the anaerobic bacteria is species of the genus *Clostridium,* and the transformed bacteria is capable of high level replication in tumor tissue *in vivo.* When injected into a subject, the genetically engineered *Clostridium* proliferate and produce the encoded enzyme in the hypoxic/necrotic

regions of tumor tissue with little or no expression in non-cancerous tissue. The tumor-bearing individual is treated systemically with a prodrug which is converted to its toxic counterpart by the enzyme which, due to the anaerobic nature of the bacteria producing it, is specifically expressed in the hypoxic/necrotic regions of

5   the tumor.

The invention also relates to recombinant bacteria comprising the coding sequence for an enzyme capable of converting a prodrug to a chemotherapeutic agent. The bacteria are transformed under conditions effective to achieve a high level of proliferation in an *in vivo* tumor. These conditions include one or more of

10 the following: (i) presence of an inhibitor of nuclease activity; (ii) a temperature of less than about 10 °C and above 0 °C; or (iii) an environment having an oxygen level that maintains growth of said anaerobe. In a preferred embodiment, all three conditions are maintained during contact of the anaerobe with the recombinant expression vector. In other preferred embodiments, the temperature is less than

15 about 5 °C but above freezing (*i.e.*, above the freezing temperature of the medium in which the anaerobe and the vector are held, typically about 0 °C).

In a preferred embodiment, *Clostridium sporogenes* the anaerobe.

Enzymes capable of catalyzing the conversion of a prodrug into a chemotherapeutic agent for expression in *Clostridium spp.* include cytosine

20 deaminase, nitroreductase, β-glucuronidase, β-galactosidase, carboxypeptidase, alkaline phosphatase and β-lactamase.

Glucuronide conjugates of epirubicin, doxorubicin, 5-fluorocytosine, CB1954, 4-hydroxycyclophosphamide or hydroxyanaline mustard, etoposide phosphate, doxorubicin phosphate, mitomycin C phosphate and 5-

25 fluorocytosineare are exemplary prodrugs.

The invention also provides a method of targeting a chemotherapeutic agent to a tumor in a tumor-bearing individual by administering to the individual an amount of recombinant *Clostridium* bacteria capable of proliferating and producing an enzyme effective to catalyze the conversion of a prodrug into a

30 chemotherapeutic agent in the hypoxic/necrotic environment of the tumor; and systemically administering a prodrug that is converted at the site of the tumor to a toxic chemotherapeutic agent by the enzyme produced by the bacteria.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

5   Brief Description of the Figures

Figure 1 illustrates the construction of a clostridial vector (pNTR500F) expressing nitroreductase (NTR).

Figure 2 illustrates the nucleotide sequence (SEQ ID NO:1) of the modified *Clostridium pasteurianum* Fd gene promoter. One of the *MboI* sites employed in 10   the original cloning of the 64 bp fragment has been bracketed. The nucleotides shown 5' to this *MboI* site, including the *EcoRI* site, are derived from M13mp7 The −35 and −10 promoter motifs are overlined, and the ribosome binding site and inserted *lac* operator motif are boxed. Nucleotides created by site directed mutagenesis are shown in lower case. The boxed ATG corresponds to that of the 15   original ferrodoxin structural gene, but becomes that of the *lacZ'* gene in all plasmids described in Figures 3-4.

Figure 3 illustrates the contruction of pMTL1006 plasmid, which is derived from pMTL1003 by replacing the *trp* promoter of the latter with the modified ferrodoxin (*Fd*) promoter. The MCS is that of pMTL20 (Chambers *et al.*). Key: 20   MCS = multiple cloning site; Ap = ampicillin; ^{R} = resistance; PAR = the partition locus of plasmid pSC101; *trp* = the *E. coli trpE* promoter; ori = origin of replication; and Fd = ferrodoxin gene promoter.

Figure 4 illustrates the construction of the expression vector pMTL500F. Following cleavage of pMTL1006 and pMTL500E with *Bg1I*, the indicated 25   fragments (A and B) were isolated from the respective plasmids and ligated together to give plasmid pMTL500F. Key: Em = erythromycin; lac po = the promoter/operator region of the *E. coli* lac operon.

Figure 5A is an artists rendering of a polyacrylamide gel showing detection of recombinant nitroreductase activity and protein in tumor homogenates. Tested 30   on the nondenaturing polyacrylamide gel were mixtures of EMT6 tumor lysate and *C. acetobutylicum* expressing nitroreductase; EMT6 tumor lysate alone; and lysate of NTR-expressing *C. acetobutylicum* alone.

Figure 5B is an artists rendering of a Western blot, stained with antibody raised against *E. coli* NTR. The lanes contained molecular weight markers;

purified nitroreductase as standard; EMT6 tumor lysates derived from tumor-bearing mice treated with saline; 10^8 spores from wild type (wt) or NTR-expressing C. acetobutylicum.

Figure 6 illustrates the cell killing of SCCVII cells in vitro exposed to
5   CB1954, NADH, and supernatants from wild type (wt) or recombinant C. acetobutylicum.

Figure 7 illustrates construction of the cloning vector pMTL540E. Plasmid pCB102 was digested with HindIII and LspI, the fragments generated blunt-ended by treatment with T4 DNA polymerase and a 1.53 Kb fragment carrying the
10   pCB102 minimal replicon regions isolated. The replicon probe vector pMTL20E was digested with NheI, blund-ended T4 DNA polymerase, and ligated to the isolated LspI-HindIII fragment. The resultant plasmid was designated pMTL540E. The arrows within the map of pCB102 correspond to the major open reading frames (ORFs) identified following translation of its nucleotide sequence, where
15   ORF H is though to play a role in replication.

Figure 8 illustrates the construction of the expression vector pMTL540F. Following cleavage of pMTL500E and pMTL540E with Bg1I, the indicated fragments (A and B) were isolated from the respective plasmids and ligated together to give plasmid pMTL540F.
20   Figure 9 illustrates construction of the expression vector pCD540FT which contains a coding sequence for expression of cytosine deaminase.

Figure 10 shows a Western blot analysis of cytosine deaminase protein expression in transformed C. sporogenes (C. sporogenes w/pCD), wild type C. sporogenes (C. sporogenes wild type) and an E. coli control transformed with the
25   same vector (E. coli w/pCD).

Figure 11 shows the percent growth of SCCVII cells as a function of drug concentration when incubated with 5-fluorouracil (open circles, 5-FC only), 5-fluorouracil (larger closed circules; 5-FU only), 5-FC plus 5, 10 or 20 μg/ml of a cell extract ("CE") from recombinant C. sporogenes (diamonds, inverted triangles and
30   triangles, respectively), or 5-FU plus 20 μg/ml of a cell extract from recombinant C. sporogenes (smaller closed circles), or 5-FU plus 20 μg/ml of a cell extract from an empty vector (open squares).

Figure 12 is a Western blot analysis of cytosine deaminase protein expression in tumor homogenates prepared from in vivo tumors injected with with

spores of cytosine deaminase-transformed *C. sporogenes.* The two left lanes are controls of an *E. coli* transformed the cytosine deminase vector [*E. coli* pCD (control)] and *C. sporogenes* transformed the CD vector [*C. sporogenes* pCD (control)].

5   Figure 13 is a Western blot analysis of cytosine deaminase protein expression in SCCVII tumors and several normal tissues (brain, heart, lung, liver, kidney, and spleen), taken from tumor-bearing mice after injection with recombinant spores of *C. sporogenes.* The two left lanes are controls of an *E. coli* transformed the cytosine deminase vector [*E. coli* pCD (control)] and *C.*
10   *sporogenes* transformed the CD vector [*C. sporogenes* pCD (control)].

Figure 14 is a plot of relative tumor volume as a function of days after injection of 10^8 spores of recombinant cytosine deaminase-transformed *C. sporogenes* on day 0 and treated with saline (control; open circles), 5-FC (open squares), 5-FU (closed triangles), recombinant spores (closed squares), or
15   recombinant spores plus 5-FC (closed circles). The data reflects the analysis of five mice per group reported as mean + standard error of the mean (SEM).

Figure 15 shows the numbers of colony forming units (CFU) of vegetative cytosine deaminase-expressing *C. sporogenes* in SCCVII tumors at 7 and 14 days after a single injection of spores in mice treated for 2 weeks with saline or with 5-
20   FC.

Detailed Description of the Invention

I.   Definitions

Unless otherwise indicated, all technical and scientific terms used herein
25   have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook *et al.,* 1989, and Ausubel FM *et al.,* 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

30   All publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention.

A "heterologous" nucleic acid construct or sequence has a portion of the sequence which is not native to the cell in which it is expressed. Heterologous,

with respect to a control sequence refers to a control sequence (*i.e.* promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell, by infection, transfection, microinjection, electroporation, or the like. A "heterologous" nucleic acid construct may contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence combination found in the native cell.

The terms "vector", as used herein, refer to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art. A cloning or expression vector may comprise additional elements, *e.g.*, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, *e.g.* in human cells for expression and in a prokaryotic host for cloning and amplification. Cloning and expression vectors will typically contain a selectable marker.

As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream gene. The promoter will generally be appropriate to the host cell in which the target gene is being expressed. The promoter together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") are necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. A promoter may be constitutive or inducible and may be a naturally occurring, engineered or hybrid promoter.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, *e.g.*, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

10

As used herein, the term "operably linked" relative to a recombinant DNA construct or vector means nucleotide components of the recombinant DNA construct or vector that are directly linked to one another for operative control of a selected coding sequence. Generally, "operably linked" DNA sequences are

5   contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

10   As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, which may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

15   Exemplary computer programs which can be used to determine identity between two sequences include, but are not limited to, the suite of BLAST programs, e.g., BLASTN, BLASTX, and TBLASTX, BLASTP and TBLASTN, publicly available on the Internet. See, also, Altschul, S.F. et al., 1990 and Altschul, S.F. et al., 1997.

20   Sequence searches are typically carried out using the BLASTN program when evaluating a given nucleic acid sequence relative to nucleic acid sequences in the GenBank DNA Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences which have been translated in all reading frames against amino acid sequences in the GenBank

25   Protein Sequences and other public databases. Both BLASTN and BLASTX are run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. [See, Altschul, et al., 1997.]

A preferred alignment of selected sequences in order to determine "% identity" between two or more sequences, is performed using e.g., the CLUSTAL-

30   W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one

another under moderate to high stringency hybridization and wash conditions. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex or probe. *E.g.*, "maximum stringency" typically occurs at about Tm-5°C (5° below the Tm of the probe); "high stringency" at about 5-10°

5   below the Tm; "intermediate stringency" at about 10-20° below the Tm of the probe; and "low stringency" at about 20-25° below the Tm. Functionally, maximum stringency conditions may be used to identify sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify sequences having about 80% or more sequence identity with

10   the probe.

Moderate and high stringency hybridization conditions are well known in the art (see, *e.g.*, Sambrook, *et al*, 1989, Chapters 9 and 11, and in Ausubel, F.M., *et al.*, 1993, expressly incorporated by reference herein). An example of high stringency conditions includes hybridization at about 42°C in 50% formamide, 5X

15   SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1X SSC and 0.5% SDS at 42°C.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or

20   that the cell is derived from a cell so modified. Thus, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention.

As used herein, the terms "transformed", "stably transformed" or

25   "transgenic" with reference to a mammalian cell means the mammalian cell has a non-native (heterologous) nucleic acid sequence integrated into its genome which is maintained through two or more generations.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The

30   process typically includes transcription and translation, however, in some cases the term "expression" may be used to refer to transcription in the absence of translation.

As used herein, the terms "high level proliferation" and "high level replication" refers to bacterial replication at a level that typically results in greater

than 10^6 and more typically greater than 10^7 bacteria per gram of tumor in a solid tumor *in vivo* in an animal. In a preferred embodiment, the term is with reference to growth of anaerobic bacteria of the genus *Clostridium* in solid tumors.

5   The term "under conditions effective to achieve" refers to the laboratory conditions of temperature, atmospheric oxygen level, and/or presence or absence of a nuclease inhibitor that are required to transform an obligate anaerobic bacteria species to yield a transformed bacteria capable of such high level proliferation.

II.   Compositions and Methods of the Invention.

10   A. Selective Infection of Tumor Cells

The ability of spores of the genus *Clostridium* to germinate in hypoxic/necrotic tissue is widely known. Tetanus and gas gangrene, for example, result from successful colonization of necrotic tissue by pathogenic strains of these organisms. In the absence of necrotic tissue, however, these organisms are inert.

15   In 1955, Malmgren and Flanagan, showed that tumor-bearing mice died of tetanus within 48 hours of intravenous injection of *C. tetani* spores, whereas non tumor-bearing animals were unaffected. Microscopic observations confirmed that vegetative forms were localized solely in the tumor tissue. Möse *et al.*, 1959 and 1964, later reported that a nonpathogenic clostridial strain, *C. butyricum* M-55,

20   localized and germinated in solid Ehrlich tumors, causing extensive lysis without concomitant effect on normal tissues. These observations have been confirmed and extended by a number of investigators using tumors in mice, rats, hamsters, rabbits, and man (Carey *et al.*, 1967; Engelbart *et al.*, 1964; Thiel *et al.* 1964).

It was a common finding, particularly with the very large tumors used in the

25   experiments performed in the early 1960's, that the extensive lysis of a tumor often produced death of the animal, presumably due to toxins released into the blood stream from the clostridia or the lysed cells. The present invention does not require cellular lysis for activity. In fact, it is advantageous to stop the reaction before extensive cellular lysis has occurred.

30   Intravenously injected clostridial spores have been demonstrated to exhibit specificity for tumors by localization to, and germination in, the hypoxic/necrotic tissue of tumors. The feasibility of clostridial-directed enzyme prodrug therapy (CDEPT) was demonstrated by cloning the *Escherichia coli* B gene encoding nitroreductase (which converts the prodrug CB1954 to a highly toxic bifunctional

alkylating agent) into a clostridial expression vector and introducing the resultant plasmid into *Clostridium beijerinckii* (formerly *C. acetobutylicum*) NCIMB 8052 (Minton *et al.*, 1995).

5   B.   Genetically-Engineered Anaerobic Microorganisms

       1. Nitroreductase expressing *C. acetobutylicum* (a.k.a. *C. beijerinkii*)

       Nonpathogenic strains of clostridia which allow genetic manipulations include, but are not limited to anaerobic bacteria, particularly members of the genus *Clostridium*. In exemplary embodiments of the present invention, the

10   bacteria is *C. acetobutylicum* (later renamed *C. beijerinkii*) or *C. sporogenes* (of which *C. oncolyticum,* previously known as *C. butyricum,* is a member).

       In studies in support of the invention, *C. acetobutylicum* (later renamed *C. beijerinkii*) was used, as will be described below. *C. acetobutylicum* has a long history, dating back to World War I for use in producing acetone and butanol, and

15   more recently has been manipulated molecularly to exploit its biotechnological potential (Minton *et al.*, 1993). *C. acetobutylicum* germinates in tumor tissue, and tumor lysis has been reported for this strain (Mose *et al.*, 1964). Antitumor activity is optimally produced by spores rather than the vegetative form (Mose and Mose, *supra*), and *C. acetobutylicum* produces spores under the conditions present in the

20   targeted tumor. The nonpathogenic clostridial strain, *C. acetobutylicum* (*C. beijerinkii* strain NCIB 8052), is exemplified herein to demonstrate the basis for the present invention.

       As described in Example 1, *Clostridium beijerinkii,* was genetically engineered to produce the *E. coli*-derived *nitroreductase* gene in experimental

25   tumors in mice, (see also, Lemmon *et al.*, 1997). A recombinant plasmid capable of directing the expression of the *E. coli* nitroreductase (*ntr*) gene in *C. acetobutylicum* (later renamed *C. beijerinkii*) was constructed, as described in detail in Example 1 and shown in Figs. 1-4. In essence, a *Nde1* site was created 'over' the translational start codon of the *ntr* gene allowing its subsequent insertion

30   into pMTL500F immediately adjacent to the clostridial ferrodoxin (Fd) promoter and ribozyme binding site (RBS).

Following construction, the vector pNTR500F was transformed into *B. substilis* and *C. acetobutylicum* for testing *in vitro* and *in vivo*. In *vitro* expression of the recombinant *E. coli* NTR in bacterial cultures was quantified using a spectrophotometric enzyme activity assay that measured NTR activity in

5 supernatant and cell pellet fractions of transformed clostridial cultures. This assay, based upon the reduction of dichlorophenol-indophenol in the presence of NADH, demonstrated that clostridia transformed with the plasmid containing the *E. coli ntr* gene (pNTR500F) exhibited a 200-fold increase in NTR activity when compared to the endogenous activity of the parental wild-type strain in mid-log phase cultures.

10 In other *in vitro* tests, *E. coli* NTR activity was visualized in nondenaturing polyacrylamide gels and by Western blot, and the results are shown in Figs. 5A and 5B. The results demonstrated that *C. acetobutylicum* spores were able to facilitate the expression of recombinant NTR *in vivo* and that recombinant NTR could be detected in tumor homogenates.

15 The ability of recombinant *C. acetobutylicum* to activate CB1954 was also tested *in vitro*, also as described in Example 1. Briefly, recombinant and wild type *C. acetobutylicum* were inoculated into medium, and after overnight incubation, the cultures were centrifuged and the supernatant collected. The supernatant was added to cultures of SCCVII tumor cells, along with addition of various

20 concentrations of CB1954 in DMSO. After incubation for two hours, the cells were counted by hemocytometer, and appropriate dilutions plated and incubated for colony growth. The results are shown in Fig. 6, and show that the supernatant from the transformed *C. acetobutylicum* was capable of enhancing the toxicity of CB1954 by approximately 10-fold.

25 In *vivo* studies with three different transplanted mouse tumors, EMT6, SCCVII and RIF1 tumors and with human colon carcinoma HT29 transplanted into immune-deficient SCID mice were performed, also as described in Example 1. Tumors were injected with spores of control and recombinant *C. acetobutylicum* when the subcutaneous tumors reach a diameter of between approximately 0.5

30 and 1 cm. The prodrug CB1954 was injected into the tumor-bearing mice at different times during the growth of the *C. acetobutylicum* and the response of the tumor was measured by counting the total clonogenic cells per tumor 24 hrs after treatment and by regrowth delay. Upon analysis, it was observed that the level of

viable clostridia in the tumors were approximately 10^5 to 10^6 bacteria per gram of tumor.

Together, the *in vitro* and *in vivo* studies established that an active foreign enzyme can be selectively expressed in tumors without detectable expression of

5   the exogenous enzyme in normal tissues. This is accomplished following intravenous injection of the inactive spore form of the bacteria, exemplified by recombinant *C. beijerinckii*, which upon transition to a reproductive state, expressed the *E. coli* nitroreductase gene.

10   2. Cytosine Deaminase-expressing *C. sporogenes*

In other studies performed in support of the invention, *C. sporogenes* was transformed with a gene for expression of cytosine deaminase. Prior to the present invention, attempts to transform *C. sporogenes* (of which *C. oncolyticum* is a member) were unsuccessful and the literature lacked information as to how to

15   transform strains of *C. sporogenes*. As will be described, an expression vector was constructed and an electroporation technique was developed that resulted in successful transformation of *C. sporogenes*. Further, in studies where transformed spores were injected into mice, it was demonstrated that *C. oncolyticum* and its closely related strain *C. sporogenes* gave 10^8 to 2×10^8

20   bacteria/g of tumor, a level of bacterial replication that is practical for clinical application of the present invention.

C. Vector Construction and Transformation

Construction of the expression vector is described in Example 2A. Briefly,

25   , as illustrated in Fig. 7 plasmid pCB102 was digested with *HindIII* and *LspI*. The fragments generated were blunt-ended by treatment with T4 DNA polymerase and a 1.53 Kb fragment carrying the pCB102 minimal replicon regions was isolated. The replicon probe vector pMTL20E was digested with *NheI*, blunt-ended T4 DNA polymerase, and ligated to the isolated *LspI-HindIII* fragment. The resultant

30   plasmid, shown in Fig. 7, was designated pMTL540E. The arrows within the map of pCB102 correspond to the major open reading frames (ORFs) identified following translation of its nucleotide sequence, where ORF H is though to play a role in replication.

16

Next, as shown in Fig. 8, plasmids pMTL500E and pMTL540E were cleaved

with *Bg1I*. The indicated fragments (A and B) were isolated from the respective

plasmids and ligated together to give plasmid pMTL540F. The *E. coli* cytosine

deaminase gene was inserted into the pMTL540F plasmid as described in

5   Example 2A. The cytosine deaminase containing plasmid is shown in Fig. 9 and is

referred to herein as pCD540FT and is abbreviated in some figures as "pCD".

C. *sporogenes* bacterial cells were transformed with the DNA plasmid

vector pCD540FT by the electroporation procedure described in Example 2B. As

noted above, until the present invention *Clostridia ssp.* have been difficult to

10   transform to obtain expression of enzymes at a level sufficient to activate an anti-

tumor prodrug. Technical obstacles relating to the extracellular secretion of

endonucleases by the clostridia and relating to maintaining a "cold", oxygen-free or

nearly oxygen free environment during transformation contribute to the difficulty of

successful transformation. Successful transformation as used herein refers to

15   transformation of the anaerobe to obtain expression of an enzyme capable of

activating an anti-tumor prodrug at a clinically useful level, *e.g.,* a "high level

proliferation" of the transformed bacteria.

The transfomation procedure described herein overcomes these technical

obstacles to achieve a high level of proliferation in a tumor. As detailed in

20   Example 2B, C. *sporogenes* was transformed under conditions effective to achieve

a high level proliferation. These conditions included at least one of (i) the

presence of an inhibitor of nuclease activity; (ii) a temperature of less than about

10 °C but above freezing of the medium containing the bacterial cells and the

transformation vector, which is typically above about 0 °C; and (iii) an environment

25   having an oxygen level that allows for or maintains growth of the anaerobic

bacteria.

With respect to condition (i), the inhibitor of nuclease activity can be an

inhibitor of endonucleases. That is, the inhibitor is capable of preventing the

endonuclease activity of hydrolysis of interior bonds of ribonucleotide or

30   deoxyribonucletide chains. An exemplary inhibitor is aurintricarboxylic acid.

Those of skill in the art are readily able to determine the amount of inhibitor

required for effective inhibition.

With respect to condition (ii), the temperature for the transformation is

maintained at less than about 10 °C but above freezing of the transformation

medium, such as an electroporation buffer in which the bacterial cells and the recombinant vector are contacted. More preferably, the temperature is maintained at less than about 5 °C, but above freezing. It will be appreciated that the freezing temperature will vary according to the components of the medium, but is typically

5   about 0 °C. In a more preferred embodiment, the temperature is less than about 4 °C but above freezing of the transformation medium.

With respect to condition (iii), the environment in which the transformation is conducted is controlled such that the oxygen level permits growth of the anaerobe. Such a "low-level" oxygen environment can be maintained by use of a hypoxic

10   chamber or other suitable controlled environment. An exemplary "low level" oxygen environment is one having less than about 100 ppm oxygen, more preferably less than about 50 ppm oxygen, and most preferably less than about 10 ppm oxygen.

In the procedure described in Example 2B, C. sporogenes was successfully

15   transformed under conditions that included (i), (ii) and (iii). More specifically, the DNAase inhibitor aurintricarboxylic acid was included in the transformation medium, an electroporation buffer; the temperature of the medium was maintained at less than about 4 °C and the surrounding atmosphere was controlled to be nearly oxygen free. The cold temperature and the low level oxygen were provided

20   by the dry ice container described in Example 2B.

The transformation procedure involves contacting the anaerobic bacteria cells to be transformed with the recombinant expression vector. The cells and the vector can, for example, be brought into contact in a suitable transformation medium, such as an electroporation buffer. In one embodiment, contact of the

25   cells and the vector is combined with electroporation to facilitate introduction of the vector into the cell.

D. *In vitro* Expression and Activity of Cytosine Deaminase

Fig. 10 shows Western blot analysis of cytosine deaminase protein

30   expression in cell lysates of transformed C. sporogenes ("C. sporogenes w/pCD"), wild-type C. sporogenes, and E. coli transformed with the same vector ("E. coli w/pCD"). The blot clearly shows expression of cytosine deaminase by the transformed baceteria and an absence of cytosine deaminase in the wild type bacteria.

The function of the *C. sporogenes*-derived cytosine deaminase was tested by assaying its ability to convert the nontoxic 5-fluorocytosine (5-FC) to the toxic 5-fluorouracil (5-FU). Fig. 11 shows data for growth inhibition of SCCVII cells incubated with 5-FC alone (open circles), 5-FU alone (larger closed circles), or in

5 the presence of various concentrations of cell extract ("CE" in the figure legend; "CE" concentrations of 5, 10, 20 μg/ml) from recombinant *C. sporogenes*. The results show that the cell extract from recombinant *C. sporogenes* expressing cytosine deaminase increases the cytotoxicity of 5-FC by a factor of 10^4, bringing it to the same toxicity as 5-FU (larger closed circles) in the assay. Cell extract from

10 *C. sporogenes* transformed with an empty vector did not affect the sensitivity of 5-FC (open squares), nor did cell extract from recombinant *C. sporogenes* affect sensitivity to 5-FU (smaller closed circles).

E. *In vivo* Expression of Cytosine Deaminase and Antitumor Activity

15 The expression and activity of cytosine deaminase was evaluated in SCCVII tumors in mice, as described in Example 2. Tumor-bearing animals were injected with 10^8 spores of recombinant *C. sporogenes*, prepared as described above. The tumors were excised 1-2 weeks later and samples prepared for immunoblot analysis of cytosine deaminase and for cytosine deaminase activity *in*

20 *vitro* as judged by the ability to convert 5-fluorocytosine to a more toxic product. Western blots of homogenates of SCCVII tumors taken 14 days after injection of the cytosine deaminase-transformed *C. sporogenes* are shown in Fig. 12. The left panels are cell lysates transfected with vectors of cytosine deaminase-transformed *E. coli* and of cytosine deaminase-transformed *C. sporogenes* as controls. The

25 two right panels Fig. 12 show that cytosine deaminase can be detected by immunoblot analysis in these tumors.

It was further demonstrated that incubating SCCVII cells with 5-FC plus tumor extract for two days resulted in an increase in cytotoxicity of 5-FC by a factor of approximately 10^3.

30 In addition, SCCVII tumor-bearing mice were injected intraveneously with 10^8 spores of recombinant *C. sporogenes,* followed by removal of the tumor, brain, heart, lung, liver, kidney, and spleen tissue seven days later. Immunoblot analysis was performed on equal amounts of protein from each of these tissue sources, and the results are shown in Fig. 13. The two left lanes in Fig. 13 correspond to

cell extracts from cytosine deaminase-transformed *E. coli* and *C. sporogenes* as control. As seen in the blot, cytosine deaminase protein was confined to the tumor tissue, with no measurable cytosine deaminase protein found in the brain, heart, lung, liver, kidney, or spleen.

5   In another *in vivo* study, the ability of recombinant cytosine deaminase-expressing *C. sporogenes* to produce sufficient conversion of 5-FC to 5-FU *in vivo* to produce antitumor activity was evaluated. As described in Example 2C, tumor-bearing mice with an average tumor size 150 mg were injected with saline (control; open circles), 5-FC (open squares), 5-FU (closed triangles), recombinant spores

10   alone on day 0 (closed squares), and recombinant spores one day prior to daily injections of 5-FC (closed circles). The results are shown in Fig. 13. The combination of 5-FC plus recombinant spores (closed circles) produced a similar or even greater delay in tumor growth than that produced by a maximum tolerated dose of 5-FU (closed triangles) given on the same schedule. The results establish

15   the antitumor efficacy of tumor-specific targeting of an anticancer drug delivery system using an obligate anaerobe.

F. Persistence of Clostridial Colonization during Therapy

          It has previously been observed that following transfection of tumor cells with prodrug-activating enzymes, the transduced cells may be rapidly and

20   selectively killed and leave the population during therapy (Lawrence *et al.*, 1998). If such rapid and selective killing were to take place, it would very difficult to give multiple treatments and particularly in the case of cytosine deaminase conversion of 5-FC to 5-FU, to maintain adequate levels of 5-FU as a radiosensitizer during

25   fractionated radiotherapy (Lawrence *et al.*, 1998). However, given that anticancer drugs are typically less active against microorganisms, it was hypothesized that this would not be a problem. This was confirmed by measuring the number of clostridial colony-forming units (CFU) per gram of tumor on day 7 and day 14 after administering spore alone or spores plus 5 times per week injections of 5-FC (500

30   mg/kg/day). As can be seen in Fig. 15, there was no significant difference between the two groups, and levels of clostridial CFUs were undiminished at day 14 as compared to day 7.

          The studies described herein demonstrate the effective expression of recombinant enzymes that are capable of converting prodrugs into

chemotherapeutic agents by recombinant *Clostridium* bacteria. A high level replication of viable clostridia in the hypoxic environment of solid tumors resulting in localized expression of the recombinant enzyme nucleic acid was achieved, along with a corresponding localized conversion of the prodrug into the active chemotherapeutic agent. The studies also show a significant antitumor efficacy of systemically injected 5-fluorocytosine following injection of recombinant spores. This demonstrates that systemically administered recombinant *Clostridium* can be used to achieve tumor specific expression of a prodrug-activating enzyme resulting in anti-tumor activity.

III.   Tumor Treatment Strategies

In order to improve the efficacy of current chemotherapeutic treatment and diminish side effects, prodrug strategies have evolved in an attempt to achieve site-specific delivery of cytotoxic anticancer agents. This strategy is based on the relative lack of toxicity of prodrugs in comparison to their anticancer (chemotherapeutic) drug counterparts. Such strategies often take advantage of differences between tumor cells and "normal" healthy cells, based on tumor-associated factors, such as hypoxia, tumor-associated enzymes and receptors.

Human solid tumors, which make up more than 90% of all human cancers are known to comprise a hypoxic environment. This leads to resistance to radiotherapy and anticancer chemotherapy, as well as predisposing to increased tumor metastases (Gray *et al.*, 1953; Hockel *et al.*, 1996; Brizel *et al.*, 1996).

Recent cancer treatment strategies have focused on the differences between tumor cells and normal healthy cells in drug targeting. It is generally known in the art that oxygen deficiency in the core of solid tumors leads to enhanced activity of reducing enzymes, *e.g.*, nitroreductases, which can be used for site-specific conversion of prodrug to drug.

Hypoxia is unique for human solid tumors and non-pathogenic spore-forming anaerobic bacteria of the genus *Clostridium* have been used experimentally as anti-cancer agents because of their selective germination and proliferation in hypoxic regions of solid tumors after systemic injection of the inactive spore form of the bacteria. The present invention takes advantage of the unique properties of tumor cells in tumor-specific enzyme/ prodrug gene therapy using genetically engineered species of anaerobic organisms, exemplified herein

by species of *Clostridia*. In a preferred embodiment of the present invention, the clostridial strain is designated as *Clostridium sporogenes*, *Clostridium oncolyticum* or *Clostridium butyricum* strain (later renamed *C. oncolyticum*).

5   Any of a number of non toxic prodrugs may be converted to a toxic chemotherapeutic agent by the action of an enzyme. Exemplary enzymes that may be used to convert a prodrug into the form of an active chemotherapeutic agent include, but are not limited to, cytosine deaminase, nitroreductase, β-glucuronidase, β-galactosidase, a carboxypeptidase, alkaline phosphatase and β-lactamase. See, *e.g.*, Melton *et al.*, 1996.

10   For example, β-glucuronidase may be used to convert prodrugs comprising glucuronides such as epirubicin, doxorubicin, or 4-hydroxycyclophosphamide into forms which are active chemotherapeutic agents.

Some examples of such prodrugs and corresponding toxic chemotherapeutic agents include the following:

15

A. Nitroreductase with CB1954

CB1954 as used herein refers to the monofunctional alkylating agent (5-[aziridin-1-yl]-2,4-dinitrobenzamide) and to analogues thereof (Wilson *et al.* 2002). The compound was was synthesized almost 25 years ago and shown to have potent
20   activity against the Walker 256 rat carcinoma *in vivo* (Cobb *et al.* 1969) and on Walker carcinoma cells *in vitro* (Roberts *et al.*, 1986). However, it has been shown to exhibit poor activity against a range of mouse and human tumor cell lines (Boland *et al.*, 1991; Workman *et al.*, 1986). The extreme toxicity of the drug to Walker tumor cells was demonstrated to result from its conversion to a bifunctional alkylating agent
25   which caused DNA interstrand crosslinks in these cells (Roberts *et al.*, *supra*). Subsequently, the toxic bifunctional alkylating agent was identified as the 4-hydroxylamine metabolite of CB1954, which was formed by a DT-diaphorase enzyme in Walker carcinoma cells (Knox *et al.*, 1988). The difference in sensitivity between rat and human cells (a factor of 10^4 - 10^5) was shown to be the result of
30   differences in the rates of reduction of CB1954 by rat and human DT diaphorases: the human form of DT diaphorase is intrinsically less able to reduce the compound than the rat form (Boland *et al.*, *supra*).

The 4-hydroxylamine toxic metabolite can be formed in one cell and can freely diffuse to produce cytotoxicity in another, as demonstrated by Knox *et al.*,

1988). It has been shown that the toxic metabolite can diffuse through clostridial cell walls and, therefore, the toxic species can be formed within the nitroreductase-expressing recombinant. This obviated the need for external NADH or NADPH co-factors, since *C. acetobutylicum* possesses both of these electron donors

5   (Petitdemange *et al.*, 1976). This is an advantage over the ADEPT approach, in which the enzyme is located outside of the cell requiring application of the co-factor. The nitroreductase enzyme is also found in the supernatant of recombinant *C. acetobutylicum*, demonstrating that the recombinant enzyme is present within and outside the bacteria.

10   One enzyme particularly preferred for expression in recombinant *Clostridium*, is the nitroreductase enzyme from *E. coli* which was recently isolated and shown to reduce CB1954 at a rate 60 times greater than that of the rat DT diaphorase. (Anlezark *et al.*, 1992).

15   B. β-Glucuronidase With Glucuronidated Anticancer Drugs
         The glycosidase β-glucuronidase occurs in both prokaryotic and eukaryotic organisms. Its concentration in human serum is very low (Stahl *et al.*, 1984), and, therefore, glucuronide prodrugs are relatively stable in blood following IV administration. Indeed, the conjugation of xenobiotics, including anticancer drugs,

20   with glucuronide is a major route by which such drugs are eliminated (Connors *et al.*, 1971-1980, 1973; Maftouh *et al.* 1984; Weenen *et al.*, 1984).
         Wang *et al.*, 1992 conjugated β-glucuronidase from *E. coli* to MAb RH1, a murine IgG that binds strongly to rat hepatoma cells, but not to human hepatoma cells. As a prodrug they tested a butylated ammonium salt of the glucuronide

25   conjugate of hydroxyanaline mustard. They found that the prodrug was 10^3 times less potent than hydroxyanaline mustard (HAM) and showed that when β-glucuronidase, either free or conjugated to the MAb, was added to the prodrug, it became as toxic as the nonglucuronide form, HAM. See also, Haisma *et al.*, 1992 who conjugated *E. coli*-derived β-glucuronidase to the anti-pan carcinoma, MAb

30   323/A3. As a prodrug, they used the glucuronide conjugate of epirubicin, an anticancer agent used in patients with breast cancer, lymphomas, ovarian cancer, and soft tissue sarcomas. They showed that the prodrug was approximately 100-fold less toxic to human breast and ovarian cancer cells, but became as toxic as epirubicin when combined with a Mab-β-glucuronidase conjugate.

A doxorubicin prodrug (N -[4-doxorubicin- N -carbonyl (oxymethyl) phenyl] O -beta-glucuronyl carbamate) has also been described for specific activation by human β-glucuronidase or β-galactosidase, which are released in necrotic areas of tumor lesions (Houba *et al.,* 1999; Houba *et al.,* 2001; Leenders *et al.,* 1999).

5

C. Cytosine Deaminase and 5-Fluorocytosine

5-Fluorouracil, an antimetabolite, has been in clinical use since 1957. Sensitive tumors include breast cancer, gastrointestinal malignancies, and cancers of the head and neck. Topical administration is useful for basal cell carcinomas

10   and other malignant dermatoses. Metabolism of 5-fluorouracil produces two critical intermediates: fluoro-uridine-5'-phosphate (FUTP), which is incorporated into RNA and interferes with its function, and fluoro-deoxyuridylate (FdUMP) which prevents normal DNA replication. However, the clinical effectiveness of 5-Fluorouracil and related compounds has been limited by toxicity, particularly

15   evident in the bone marrow.

The 1-*N*-β-glucuronide of 5-fluorouracil has been synthesized (Alexander *et al.,* 1991; Kulinkovich *et al.,* 1983) and shown to be stable *in vivo* (Germane *et al.,* 1987). It is cleaved to 5-fluorouracil by β-glucuronidase.

Cytosine deaminase catalyzes the deamination of cytosine to uracil.

20   Mammalian cells do not ordinarily produce this enzyme, whereas many bacteria and fungi do. Microorganisms that express cytosine deaminase can convert 5-fluorocytosine to 5-fluorouracil, a highly toxic metabolite that has potent cytotoxic effects on mammalian cells and is widely used as a cancer chemotherapeutic agent (Mullen *et al.,* 1992).

25   The enzyme/prodrug combination of cytosine deaminase/5-fluorocytosine has been explored using antibody-directed enzyme prodrug therapy (ADEPT) and virus-directed enzyme prodrug therapy (VDEPT) approaches, but has met with limited success (Fox *et al.,* 1996).

30   ### D. Proteases

The invasive and metastatic activities of tumor cells provide a need for tumor-associated proteases, *e.g.,* plasmin. Prodrugs of daunorubicin and doxorubicin designed for selective activation by the serine protease plasmin have been described (de Groot *et al.,* 2000 and de Groot *et al.,* 1999).

In addition, at least two carboxypeptidases have been used to convert a prodrug into a chemotherapeutic agent, *e.g.* the conversion of methotrexate-α-alanine to methotrexate (Vitols *et al.*, 1989; Vitols *et al.*, 1995). See, also Niculescu-Duvaz *et al.*, 1999, which describes the use of carboxypeptidase G2 in
5   a gene-directed enzyme prodrug therapy (GDEPT) system for use in the conversion of nitrogen mustard prodrugs into the corresponding active form. Carboxypeptidase G2 finds utility in the cleavage of amidic, urethanic or ureidic bonds between a benzene nucleus and L-glutamic acid.

10   E. Alkaline Phosphatase
Alkaline phosphatase may be used to remove the phosphate group from inactive prodrugs converting them into chemotherapeutic agents. For example, alkaline phosphatase has been used in the conversion of etoposide phosphate, doxorubicin phosphate and mitomycin C phosphate into etoposide, doxorubicin
15   and mitomycin C, respectively (Senter *et al.*, 1989).

        F. β-Lactamase
The use of antibody-directed catalysis (ADC) for the delivery of a cephalosporin derivative of the oncolytic agent 4-desacetylvinblastine-3-
20   carboxhydrazide conjugated to F(ab')-β-lactamase conjugates exhibiting both β-lactamase activity and immunoreactivity toward carcinoembryonic antigen (CEA) was shown to have potential for immunochemotherapy in preclinical models (Meyer *et al.*, 1993).

25   IV.   Expression Of Recombinant Enzymes in Anaerobic Bacteria
The present invention is based on transformation of bacteria with recombinant expression vectors effective to express an enzyme capable of converting a prodrug into a toxic chemotherapeutic agent, particularly in obligate anaerobes of the genus *Clostridia*.
30   It will be understood that while particular enzyme prodrug combinations are exemplified herein, the invention contemplates expression vectors comprising the coding sequence for any of a number of enzymes capable of converting a prodrug into a toxic chemotherapeutic agent so long as the enzyme can be expressed in

Clostridium sporogenes (of which *C. oncolyticum,* previously known as *Clostridium butyricum,* is a member).

Exemplary enzymes include, but are not limited to cytosine deaminase, nitroreductase, β-glucuronidase, β-galactosidase, a carboxypeptidase, alkaline
5   phosphatase and β-lactamase.

A. Enzyme-Encoding Nucleic Acid Sequences.

Accordingly, although specific enzyme-encoding nucleic acid sequences are exemplified herein, the invention includes homologues of the exemplified
10   coding sequences isolated from other species, naturally occurring allelic and splice variants, nucleic acid fragments, and biologically active (functional) derivatives thereof, such as, nucleic acid sequences that encode amino acid sequence variants of the native enzyme and sequences which encode fusion proteins, so long as the enzyme maintains its biological activity (the ability to convert a prodrug
15   into a toxic chemotherapeutic agent).

Due to the inherent degeneracy of the genetic code, nucleic acid sequences other than the native form, such as the *E. coli* cytosine deaminase sequence presented at GenBank Accession No. S56903, and which encode substantially the same or a functionally equivalent amino acid sequence may be used to clone
20   and/or express cytosine deaminase in *Clostridium sporogenes.* Thus, for a given enzyme-encoding nucleic acid sequence, it is appreciated that as a result of the degeneracy of the genetic code, a number of coding sequences can be produced that encode a protein having the same amino acid sequence. For example, the triplet CGT encodes the amino acid arginine. Arginine is alternatively encoded by
25   CGA, CGC, CGG, AGA, and AGG. Therefore it is appreciated that such substitutions in the coding region fall within the nucleic acid sequence variants covered by the present invention. Any and all of these sequence variants can be utilized in the same way as described herein for the native form of the nucleic acid sequence that encodes an enzyme capable of converting a prodrug into a toxic
30   chemotherapeutic agent when expressed in an obligate anaerobe.

In general, the coding sequence for particular enzymes that are effective in converting a prodrug into a chemotherapeutic agent and are therefore useful in practicing the present invention may be found in GenBank or in the scientific literature. Such enzymes may be found in one or more forms and may be isolated

from one or more species. By way of example, the *E. coli* cytosine deaminase

sequence may be found at GenBank Accession No. S56903 and a yeast derived

form of cytosine deaminase may be found at GenBank Accession No. GenBank

Accession No. U55194. Either of these coding sequence may be incorporated into

5   an expression vector for transformation into *Clostridium spp.* in practicing the

present invention. While both sequences may be effective to express cytosine

deaminase in recombinant *Clostridium*, it will be understood that in some cases

different coding sequences for the same enzyme may result in different expression

levels of a biologically active form of the enzyme. See, *e.g.*, Hamstra *et al.*, 1999.

10   In one general embodiment, such sequence variants have at least 70%,

preferably 80%, 85%, 90%, 95%, 98%, or more sequence identity to the coding

sequence for a native form of the enzyme.

In another embodiment, the enzyme-encoding nucleotide sequence will

hybridize under moderate to high stringency conditions to a nucleotide sequence

15   that encodes a native form of the enzyme.

As will be further understood by those of skill in the art, in some cases it

may be advantageous to produce nucleotide sequences possessing non-naturally

occurring codons. Codons preferred by a particular host can be selected, for

example, to increase the rate of protein expression or to produce recombinant

20   RNA transcripts having desirable properties, such as a longer half-life, than

transcripts produced from the naturally occurring sequence. Hence, a native

enzyme-encoding nucleotide sequence may be engineered in order to alter the

coding sequence for a variety of reasons, including but not limited to, alterations

which modify the cloning, processing and/or expression of the enzyme by an

25   anaerobic bacterial cell, *e.g.*, a *Clostridium* bacteria.

Particularly preferred are nucleic acid substitutions, additions, and deletions

that are silent such that they do not alter the properties or activities of the enzyme

following expression in recombinant bacteria.

The variations can be made using methods known in the art such as

30   oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR

mutagenesis. Site-directed mutagenesis [Carter *et al.*, 1986; Zoller *et al.*, 1987],

cassette mutagenesis [Wells *et al.*, 1985], restriction selection mutagenesis [Wells *et

al.*, 1986] or other known techniques can be performed on the cloned DNA to

produce the enzyme-encoding variant DNA.

B. <u>Vectors For Transformation of *Clostridium spp*</u>

Vectors for transformation of *Clostridium spp.* were constructed using the *E. coli Clostridium* shuttle vectors, pMTL500F and pMTL500FT (Minton *et al.*, 1981). These shuttle vectors are based on the previously constructed *E. coli* cloning

5   vector, pMTL500E (Oultram *et al.*, 1988). The promoter and RBS of the *lacZ'* were replaced in pMTL500F by the equivalent signals of the ferredoxin (Fd) gene of *Clostridium pasteurianum*.

Since the promoter elements and ribosome binding sites of Gram-negative bacteria are generally poorly utilized in Gram-positive bacteria, preferred vectors

10   for practicing the present invention include the multiple cloning site (MCS) placed 3' to the promoter and RBS of *Clostridium pasteurianum*, such that heterologous genes inserted into the MCS are efficiently expressed in both Gram-positive and Gram-negative bacteria. Such vectors are preferred for the expression of the nucleic acid sequence encoding an enzyme capable of converting a prodrug into a

15   chemotherapeutic agent in *Clostridium spp.*, in particular in *Clostridium sporogenes*. An Nde1 site is the first restriction site of the MCS and serves as the insertion site for the coding sequence the enzyme

The methods of the invention rely on the use of anaerobic *Clostridium* cultures to express an enzyme capable of converting a prodrug into a toxic

20   chemotherapeutic agent, in particular in *Clostridium sporogenes*, with no particular method of enzyme expression required. In this aspect , the invention provides *Clostridium* host cells that have been transduced, transformed or transfected with an expression vector comprising an enzyme-encoding nucleic acid sequence. The culture conditions, such as temperature, pH and the like, are those previously used

25   for the culture of the bacteria prior to transduction, transformation or transfection and will be apparent to those skilled in the art.

Various methods may be employed for delivering the expression vector into bacterial cells *in vitro*. Methods of introducing nucleic acids into bacteria include, but are not limited to, electroporation; microinjection; bacterial protoplast fusion

30   with intact cells; use of polycations, *e.g.*, polybrene or polyornithine; membrane fusion with liposomes, lipofectamine or lipofection-mediated transfection; high velocity bombardment with DNA-coated microprojectiles; incubation with calcium phosphate-DNA precipitate; DEAE-Dextran mediated transfection; infection with modified viral nucleic acids; and the like. In a preferred embodiment of the present

invention, electroporation is carried out under the conditions specified in Example 2.

V.   Methods of Detecting Gene Expression

5   In order to evaluate the expression of an enzyme by bacterial cells that have been transformed with an expression vector such as described herein, assays can be carried out at the protein level, the RNA level and/or by use of functional bioassays particular to the activity of the enzyme expressed by the recombinant bacteria.

10   Such assays include Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), RT-PCR (reverse transcriptase polymerase chain reaction), or *in situ* hybridization, using an appropriately labeled probe (based on the nucleic acid coding sequence) and conventional Southern blotting.

15   Alternatively, protein expression may be evaluated by immunological methods, such as immunohistochemical staining of cells, tissue sections or immunoassay of tissue culture medium, *e.g.*, by Western blot or ELISA. Such immunoassays can be used to qualitatively and quantitatively evaluate expression of a given enzyme. Exemplary methods are briefly described below, given that

20   such methods are generally known to those of skill in the art and many reagents for practicing the methods are commercially available.

          A purified form of the enzyme expressed by the recombinant bacteria may be used to produce either monoclonal or polyclonal antibodies specific to the expressed protein for use in various immunoassays. (See, *e.g.*, Harlow *et al.*,

25   1988). Exemplary assays include ELISA, competitive immunoassays, radioimmunoassays, Western blot, indirect immunofluorescent assays and the like. In many cases, commercially available antibodies and/or kits may be used for immunoassay of the enzyme.

30   A. Enzyme activity assays
          ELISA Assays for Recombinant Proteins
          Levels of enzymes, *e.g.*, cytosine deaminase, nitroreductase, β-glucuronidase, β-galactosidase, carboxypeptidase, alkaline phosphatase or β-lactamase and clostridial cell wall proteins may be determined in tumor tissue,

plasma, and normal host tissue using an ELISA technique. In order to maximize the sensitivity of the assay, a non-competitive homogeneous sandwich ELISA assay such as that described by Noe et al., 1992, is typically used.

5   Briefly, in one exemplary assay format, rabbit polyclonal antibodies raised against the purified enzyme or clostridial cell wall extract is adsorbed onto 96-well plates. Serially diluted samples of plasma, cell lysates, or standards consisting of a purified form of the protein are then added to the wells. After allowing for the antigen to bind to the immobilized antibody, the wells are washed repeatedly to remove unbound protein. A biotinylated anti-enzyme polyclonal antibody is then

10   added to the well and allowed to react with the immobilized antigen. Avidin and biotinylated alkaline phosphatase are then added, the wells are washed, and the signal quantified by adding p-nitrophenyl phosphate and reading the absorbance at 405 nm using a 96-well plate reader.

In another exemplary type of assay, β-Glucuronidase activity is measured in

15   cultures of Clostridium spp. using the spectrophotometric method described by Fishman, W.H., "β-Glucuronidase" in: METHODS OF ENZYMATIC ANALYSIS (Weinheim: Verlag Chemie, Bergmeyer, H.U. and K. Gawehn, eds. 1974). Plasma or crude cell homogenates are incubated in acetate buffer pH 4.0 with 8 mM 4-nitrophenyl-β-D-glucuronoside and monitored continuously at 405 nm for the release of 4-

20   nitrophenol (ϵ_{405}=18.5 mmol^{-1}cm^{-1}). The slope of the absorbance curve is used to calculate enzymatic activity. One unit of β-glucuronidase activity is defined as the release of 1 umol 4-nitrophenol per min at 25 C and pH 4.0.

Similarly, nitroreductase activity may be measured in cultures of Clostridium spp. using a modification of the method developed by Gordon et al., 1991. Briefly,

25   samples diluted in 25 mM Tris-HCl buffer containing 0.7 mg/ml bovine serum albumin are placed in an assay mixture containing 4 µM dichlorophenol-indophenol (DCIP) and 200 µM NADH, and the rate of DCIP reduction is followed spectrophotometrically at 600 nm. Replicate samples containing 50 µM dicoumoral, a potent inhibitor of nitroreductase, are used to correct for nonspecific

30   DCIP reduction. Protein concentrations are measured with a BCA Protein Assay Kit method (Pierce) utilizing bovine serum albumin as a standard.

B. *In vivo* assays

C3H/Km mice are typically used for transplantation of RIF-1 and SCCVII/St mouse tumors; BALB/c are typically used for transplantation of EMT6/St tumors; and SCID mice are typically used for transplantation of the HT 29 human tumor.

5   Normal tissue studies are generally performed on C3H/Km mice. It will be understood that alternative strains of mice may be used so long as the tumor cells under investigation grow in the host animal.

Rodent tumors have been used extensively over the past 5-20 years (depending on the tumor), and rigorous procedures have been established for

10   passage of the cells from frozen tumor characteristics. A full description of this is given for the RIF-1 tumor (Twentyman *et al.*, 1980), and an identical practice is used for the other tumors. Full descriptions of the derivation, characteristics and handling of the rodent tumors are known in the art. (See for example, Brown *et al.*, 1979; Brown *et al.*, 1981; Hirst *et al.*, 1982; Rockwell *et al.*, 1972).

15   *In vivo* assays for anti-tumor activity may be based on any assay that provides information on the effect of a particular treatment on tumor growth. For example, an assay such as a regrowth delay assay may used to evaluate the effect of a particular treatment on the ability of a tumor to regrow *in vivo* in an animal. Regrowth assays may be performed using any tumor that will grow in an

20   animal, *e.g.*, an SCCVII/St, RIF-1 or HT 29 tumor. After treatment, tumor volumes are measured and the time required for a tumor to regrow to a size equal to 2 or 4 times the tumor volume at the time of treatment is evaluated. (See, *e.g.*, Brown JM, 1975; Brown *et al.*, 1982; Brown *et al.*, 1990; Brown *et al.*, 1979; Stone *et al.*, 1991).

25   Another exemplary type of assay is a tumor control (TCD_{50}) assay, which is based on the dose of a compound that results in tumor control in 50% of treated mice (the TCD_{50}). After treatment tumors are measured at regular intervals. In one example of this approach, after treatment tumors are measured at weekly intervals, until they exceed 4x the treatment volume or reach 120 days post-

30   treatment. At this time, the mice are sacrificed and autopsied. A mouse is recorded as "cured" if at 120 days post-treatment it does not have a tumor greater than 4 mm mean diameter. (See, *e.g.*, Brown *et al.*, 1981; Brown *et al.*, 1979; Stone *et al.*, 1991). This assay has been used in testing the MDAH/MCa4

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carcinoma and RIF-1 sarcoma and has been applied to the SCCVII, RIF-1 mouse tumors and the HT 29 human tumor.

VI.   Utility

5   The invention described herein presents a method for specific and effective delivery of a chemotherapeutic agent to a tumor. The invention provides compositions and methods for high level expression of a prodrug-activating enzyme, together with bacteria capable of high capacity proliferation in the tumor microenvironment, resulting in a tumor-specific gene delivery system which has

10   widespread utility to treatment of cancers that manifest as solid tumors.

           Specific examples are described herein, however, it will be apparent to one of ordinary skill in the art that many modifications are possible and that the examples are provided for purposes of illustration only and do not limit the invention, unless so specified.

15   All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

VII.   Examples

           The following examples further illustrate the invention described herein and

20   are in no way intended to limit the scope of the invention.

Materials and Methods

A. Mice and Tumors

           C3H/Km mice were used for transplantation of RIF-1 and SCCVII/St mouse

25   tumors; BALB/c were used for transplantation of EMT6/St tumors; and SCID mice were used for transplantation of the HT 29 human tumor. Normal tissue studies were performed on C3H/Km mice. All the mice were bred and housed within the new Research Animal Facility at Stanford University. All the tumors were assayed for cell survival in vitro by plating single cells into petri dishes.

30

B. Growth and Sporulation of _Clostridium spp_ is carried out using routine conditions generally applied by those of skill in the art, with no particular method of culture required.

5   Sporulation cultures were typically started from exponentially growing cultures. Spores were typically stored at 4°C until needed for animal injection. Spores were injected intravenously into mice in 0.1 ml volume from spore suspensions in PBS of 10^9 spores/ml.

C. Electrophoresis and Immunoblotting

10   SDS-PAGE one dimensional electrophoresis was carried out using 12% gels (Laemmli, UK, 1970). Following electrophoresis, protein was transferred to nitrocellulose sheets using a Bio-Rad semi-dry blotter at 25 V for 1 hr using the buffer system of Towbin _et al.,_ 1979. Immunoblotting was typically performed using polyclonal antibody preparations derived from rabbits and directed against _E._
15   _coli_ cytosine deaminase, nitroreductase, or β-glucuronidase.

By way of example, nondenaturing polyacrylamide gel electrophoresis was performed by the method of Davis using 10% slab gels in a Bio-Rad Mini-Protein II apparatus (Davis, J.B., 1964). Nitroreductase activity was visualized by a modification of the method of Kaplan and Beutler used to measure glutathione
20   reductase (Kaplan _et al.,_ 1968). When oxidized glutathione was omitted from the assay mixture, this method detects both DT diaphorase and nitroreductase. These two enzymes were easily differentiated by their different electrophoretic mobilities.

D. Polyclonal Antibody Production

25   Antibodies against the enzyme expressed in recombinant anaerobes in order to convert a prodrug to its chemotherapeutic counterpart, _e.g.,_ cytosine deaminase, nitroreductase, or β-glucuronidase, were used for immunoassays and immunohistochemistry, etc. to visualize the localization of expressed recombinant enzyme in tumor and tissue slices. An antibody to crude whole-clostridia
30   homogenate was produced for use with immunohistochemistry methods to augment traditional staining methods designed to detect both vegetative and spore forms of clostridia in tissue and tumor sections. Polyclonal antibodies for use in immunohistochemistry, immunoblotting, and ELISA assays were produced in rabbits using a modification of the technique published by Oberley _et al.,_ 1990.

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Due to the bacterial origin of the antigens, prior to immunization, the antigen

preparations were rendered endotoxin-free by passing them over a lipid A-binding

affinity matrix (Detoxi-Gel, Pierce). This procedure also utilized Ribi adjuvant

instead of Freund's complete and incomplete adjuvant to minimize animal

5   discomfort.

EXAMPLE 1

Expression of the *E. coli ntr* Gene in *Clostridium spp*

A. Construction of Vectors for Expression of Nitroreductase

10   The gene (*ntr*) encoding *E. coli* B nitroreductase (NTR) was cloned and its

nucleotide sequence determined. The fragment characterized was shown to carry

the *ntr* promoter in addition to the structural gene. The transcriptional initiation

signals and the ribosome binding sites (RBS) from the gram-negative *E. coli* were

replaced with Gram-positive equivalents, as follows.

15   Based on the previously constructed *E. coli Clostridium* cloning vector,

pMTL500E (Oultram *et al*), the promoter and RBS of the *lacZ'* were replaced in

pMTL500F by the equivalent signals of the ferrodoxin (Fd) gene of *Clostridium*

pasteurianum, as illustrated in Fig. 1. A 604 bp *MboI* fragment carrying the

Clostridium pasteurianum ferrodoxin gene was isolated from the genome of strain

20   ATCC 6013 and cloned into the *BamHI* site of M13mp7. The nucleotide sequence

of the cloned fragment was found to be identical to that reported by Graves *et al*.,

1985. Site-directed mutagenesis was used to change the triplet immediately

preceding the translational start codon from TTC to CAT, thereby creating a

recognition sequence for the restriction enzyme *NdeI*. A unique *HpaI* site was also

25   created within the promoter region by changing nucleotide position 132 from "A" to

"T" (Graves *et al*., 1985). Into this created site, a 25 bp blunt-ended, synthetic

DNA fragment corresponding to the operator of the *E coli lac* promoter was

inserted. The modified Fd promoter was subsequently isolated as a 200 bp

fragment, as shown in Fig. 2 (SEQ ID NO:1), following cleavage with *NdeI* and

30   *EcoRI*, and cloned between the equivalent sites of the expression vector

pMTL1003 (Brehm *et al*., 1991) to give PMTL1006, as shown in Fig. 3. The final

step involved replacing the *lac* promoter of pMTL500E with the Fd promoter of

pMTL1006. This was achieved by combining a 1.871 Kb *Bg1I* fragment isolated

from pMTL1006 with a 4,839 Kb *Bg1I* fragment isolated from pMTL500E, as shown in Fig. 4. The resulting plasmid was designated pMTL500F.

A recombinant plasmid capable of directing the expression of the *E. coli* nitroreductase (*ntr*) gene in *C. acetobutylicum* (later renamed *C. beijerinkii*) was

5   constructed as illustrated in Fig. 1. In essence, a *Nde1* site was created 'over' the translational start codon of the *ntr* gene allowing its subsequent insertion into pMTL500F immediately adjacent to the clostridial Fd promoter and ribosome binding site. Two oligonucleotide primers were employed to amplify a 700 bp fragment carrying the *E. coli* nitroreductase gene. Simultaneously, primer 1

10   created an *Nde1* site. The amplified fragment was subsequently inserted between the *Nde1* and *Pst1* sites of the expression vector pMTL500F. In the resulting recombinant, pNTR500F, the *ntr* gene was under transcriptional and translational control of the promoter (Fd) and ribosome binding site, respectifly, of the *C. pasteurianum* ferrodoxin gene. Ap' and Em' are ampicillin and erythromycin

15   resistance genes, respectively. RepD and RepE are pAMβ1-derived replication proteins, and ORI is the replication origin of ColE1.

pNTR500F was transformed into *B. substilis* and *C. acetobutylicum* NCIMB 8052, using electroporation (Oultram, 1988).

20   ## B. Nitroreductase (NTR) Activity *in vitro*

To quantify the expression of the recombinant *E. coli* nitroreductase in bacterial cultures, a spectrophotometric enzyme activity assay was used to measure nitroreductase activity in the supernatant and cell pellet fractions of transfrmed Clostridial cultures. This assay, based upon the reduction of

25   dichlorophenol-indophenol in the presence of NADH, demonstrated that Clostridia transfomed with the plasmid containing the *E. coli ntr* gene (pNTR500F) exhibited a 200-fold increase in NTR activity when compared to the endogenous activity of the parental wild-type strain in mid-log phase cultures (44 U/mg protein in culture supernatants for trasnformed *C. acetobutyicum* vs. 0.2 U/mg for the parental

30   strain). In early log phase cultures, total nitroreductase activity was low (4 U/mg protein) and greater than 95% enzymatic activity was associated with the cell pellet. As cultures progressed into stationary phase, nitroreductase activity accumulated within the culture media and was increasingly found in the supernatant fraction. By 24 hours post-inoculation, total nitroreductase activity

within the culture had risen to 105 U/mg protein, and 95% of the activity was associated with the supernatant fraction of the culture.

E. coli NTR activity was visualized in nondenaturing polyacrylamide gels. Figure 5A is an artists rendering of the gel after staining for NTR activity, where the

5   lanes were loaded with Clostridial lysate alone or EMT6 tumor lysate alone or with a mixture of the two. The mixtures were prepared by mixing 50 μg of EMT6 tumor lysate with decreasing amounts (5 μg to 250 ng) NTR-transformed C. acetobutylicum. The test mixtures were run on a native gel and then stained for NTR activity. Both bacterial NTR and mammalian DT-diaphorase were detected

10   by the staining, however, the different electrophoretic mobilities of the two enzymes allowed NTR activity from as little as 250 ng C. acetobutylicum lysate to be easily visualized in the presence of a 200-fold excess of EMT6 protein.

Western blotting was also used to detect recombinant NTR in tumor lysates. Figure 5B shows an artists rendering of a blot, stained with anti-E. coli NTR

15   antibody, containing EMT6 tumor samples removed four days following i.v. injection of saline, wild type C. acetobutylicum spores, or spores from NTR-transformed bacteria into tumor bearing mice. The antibody detects protein of approximately the appropriate size (27 kD) and mobility of the NTR protein standard in the two samples derived from tumors injected with spores expressing

20   recombinant NTR. NTR protein is not detectable in tumor samples derived from animals injected with saline or wildtype C. acetobutylicum. An additional protein (77 kD) was also detected by this antibody in all tumor samples. This blot demonstrates that C. acetobutylicum spores are vectors capable of expressing recombinant proteins in vivo and that these recombinant proteins can be detected

25   in tumor homogenates.

Recombinant E. coli nitroreductase was partially purified from transformed C. acetobutylicum using a modification of the affinity chromatography technique of Ysern and Prochaska. A preliminary isolation yielded a 100-fold purification of nitroreductase in a single affinity chromatography step from a clarified homogenate

30   of E. coli expressing the nitroreductase plasmid. The homogenate was passed over a dye affinity column (Affi-Gel Blue, Bio-Rad) and washed with increasing concentrations of NaCl. A highly enriched nitroreductase fraction was eluted from the column in a 1 M NaCl wash. Denaturing polyacrylamide gel electrophoresis of the eluted fraction followed by silver staining demonstrated at least four strongly

staining protein bands. The major constituent was estimated to have a molecular weight of 26.5 kDa, very close to the reported size of 24 kDa for *E. coli* nitroreductase (Anlezark *et al.*). Further purification steps produced a preparation of sufficient purity to use as antigen for antibody production and standards in the

5   ELISA assay, native gel electrophoresis, and immunoblotting assays.

In order to test the ability of recombinant *C. acetobutylicum* to activate CB1954, recombinant and wild type *C. acetobutylicum* were inoculated into 20 ml of medium. After overnight incubation, the cultures were centrifuged and the supernatant collected and diluted 1:3 with Waymouth's growth medium. This

10   mixture, which had a pH of approximately 6.9, was then transferred to 60 mm, plastic petri dishes containing SCCVII tumor cells, which were approximately 50% confluent. To these cultures, different concentrations of CB1954 in DMSO were added, and the amount of DMSO adjusted in all of the cultures (including the controls) to 1% DMSO. Controls consisted of cell culture with only the

15   supernatant/cell culture medium mixture. All plates received NADH to a final concentration of 500 μM.

After a 2 hr incubation in air at 37°C, the medium was removed, all the plates were washed 3 times with PBS, trypsinized, the cells counted by hemocytometer, and appropriate dilutions plated and incubated for 10 days in a

20   5% CO_2 incubator at 37OC for colony growth. The results are presented in Figure 6, and show supernatant from the transformed *C. acetobutylicum* was capable of enhancing the toxicity of CB1954 by approximately 10-fold.

C. Nitroreductase activity *in vivo*

25   *In vivo* studies with three different transplanted mouse tumors, EMT6, SCCVII and RIF1 tumors, and with human colon carcinoma HT29 transplanted into immune-deficient SCID mice were performed. Tumors were injected with spores of control and recombinant *C. acetobutylicum* when the subcutaneous tumors reach a diameter of between approximately 0.5 and 1 cm. As a function of time

30   after injection, mice were sacrificed and their tumors removed.

Homogenates of the tumors were prepared, extracts run on a nondenaturing gel and the amount of nitroreductase quantitated by Western blot. Homogenates of normal tissue showed no detectable nitroreductase protein.

D. *In vivo* Efficacy Studies

In vivo studies with three different transplanted mouse tumors, EMT6,
SCCVII and RIF1 tumors and with human colon carcinoma HT29 transplanted into
immune-deficient SCID mice were performed. Tumors were injected with spores
5   of control and recombinant *C. acetobutylicum* when the subcutaneous tumors
reach a diameter of between approximately 0.5 and 1 cm.

CB1954 was injected into the tumor-bearing mice at different times during
the growth of the *C. acetobutylicum* and the response of the tumor was measured
by counting the total clonogenic cells per tumor 24 hrs after treatment and by
10   regrowth delay. Upon analysis, it was observed that the level of viable clostridia in
the tumors were approximately 10^5 to 10^6 bacteria per gram of tumor.

EXAMPLE 2

Expression Of Cytosine Deaminase in *Clostridium spp*

15   A. Vector Construction

A plasmid vector designated pCD540F was constructed as illustrated in
Figs. 7-9. The pMTL500F plasmid described in Example 1 (Fig. 4) was modified
by replacing the pAMβ1-derived origin of replication with a replicon derived from
the *Clostridium butyricum* plasmid pCB012 (Minton, N.P. *et al.*) Plasmid
20   pMTL540F was constructed in an analogous fashion to pMTL500F. First, a similar
plasmid to pMTL500E (Fig. 4) was made, designated pMTL540E and shown in
Fig. 7, by inserting a blunt-ended 1.53 Kb *LspI-HindIII* fragment from pCB102 into
the *NheI* site, similarly blunt-ended, of the replicon cloning vector pMTL20E
(Swinfield *et al.*). The *lac* promoter of pMTL540E was then replaced with the
25   promoter of the ferrodoxin (Fd) gene by combining a 3.64 Kb *Bg1I* fragment from
pMTL540E with a 1.871 Kb *Bg1I* fragment isolated from pMTL500F, as shown in
Fig. 8. The resulting plasmid was designated pMTL540F.

Expression of heterologous genes in pMTL540F expression vectors has
been found to be optimal when the gene is inserted in at the *Nde* 1 site of the
30   multiple cloning site (MCS), which is immediately adjacent to the promoter. Since
the *E. coli* cytosine deaminase gene contains an internal *Nde* 1 site, in
constructing an expression vector for expression of cytosine deaminase in
Clostridium spp. the forward primer was designed to contain the *Ase* 1 restriction
site, which generates ends complimentary to those produced by *Nde* 1. The

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reverse primer contained a restriction site for *Xba* 1, allowing directional cloning of
the PCR product. The gene encoding cytosine deaminase was amplified from
HB101 *E. coli* genomic DNA by PCR and a product of the predicted size was gel-
purified, digested with *Ase* 1 and *Xba* 1, and ligated to *Nde* 1/*Xba* 1 digested

5   vector DNA, as further described in Austin *et al.*, 1993 and Minton *et al.*, 1981.
Ligation products were transformed into competent GBE180 *E. coli* and plasmid
minipreps performed by a standard procedures. The absence of PCR-induced
mutations in the *CD* gene was confirmed by sequencing prior to large-scale
plasmid preparation. The vector containing the cytosine deaminase gene,

10   pCD540FT, is shown in Fig. 9 and is abbreviated in some Figs. as "pCD."

B. Transformation of *C. sporogenes*

        C. sporogenes was transformed with the pCD540FT vector comprising the
coding sequence *E. coli*-derived cytosine deaminase (CD) as follows.

15   A broth, referred to as "TPGY" broth, was prepared from the following
components, with balance water.

trypticase peptone	2%	20 g/l
peptone	0.5%	5 g/l
glucose	0.1%	1 g/l
yeast extract	0.5%	5 g/l
cysteine HCl	0.1%	1 g/l

        TPGY agar was prepared with the same components as the TPGY broth,

25   with the addition of 2% agar (20 g/l).
        An electroporation buffer was prepared by mixing 10% polyethylene glycol
(MW 8,000) containing 1 mg/ml polyanetholesulfonic acid in water.
        All broths, buffers and other media were equilibrated to anaerobic
conditions prior to transformation, *i.e.*, at least 24 hours before transformation.

30   An isolate colony of *C. sporogenes* was selected and restreaked onto a
fresh TPGY agar plate two days before transformation. One day before
transformation, a single colony from the freshly restreaked plate was inoculated
into TPGY broth. Serial dilutions (up to 10^5 times dilution) in 5 ml volumes of
TPGY broth were prepared and incubated overnight. Then, 3 ml of culture from

35   the lowest dilution having discernable cell growth was used to inoculate 300 ml
(1:100 dilution) of fresh TPGY broth in an airtight centrifuge bottle. The inoculated

broth was incubated (typically for 2.5-3 hours) until the midlog phase of growth was reached, as evidenced by spectrophotometric evaluation at 600 nm of the broth for a peak OD of 0.7-0.8.

The culture bottle containing the bacterial cells was tightly capped closed and removed from the hypoxic chamber into an ice bath for at least 10 minutes before centrifugation. The bacterial culture was kept as cold as possible, but not frozen, until electroporation.

For the electroporation, a container filled with dry ice was placed in the hypoxic chamber. The following solutions were placed on support racks along one side of the dry-ice filled container: (i) a solution containing the pCD540FT plasmid vector, (ii) a solution of auritricarboxulic acid, and (iii) 50 ml centrifuge tubes of the electroporation buffer. Electroporation cuvettes were also placed in the dry-ice filled container on a support rack.

Just prior to electroporation, the bacterial culture in the culture bottle was centrifuged (6,000 g for 10 minutes) and the cells were harvested and then washed in 10 ml cold electroporation buffer. The washed cells were again harvested by centrifugation (6,000 g for 10 minutes) and then resuspended in 3 ml cold electroporation buffer additionally containing a DNAase inhibitor, aurintricarboxylic acid at 0.1 mg/ml (diluted from stock solution at 100 mg/ml).

Into a 0.2 cm inter-electrode distance cuvette, 1-5 µg of cold DNA plasmid vector pCD540FT was added along with 0.4 ml of solution containing the bacterial cells in the DNAase inhibitor-containing buffer. The vector-cell solution in the cuvette was pulsed once at 1.25 kV, 25 µFD, 100 ohms and maintained at the cold temperature for an additional 4 minutes.

The pulsed cells were transferred into 4.6 ml TPGY broth prewarmed to about 37 °C containing 25 mM $MgCl_2$ (diluted from 2.5 M stock solution) and incubated for 4 hours. The cell culture was then divided into 5 separate microfuge tubes, each containing 1ml cell culture. The cells were harvested by centrifugation with a compact microcentrifuge placed inside the hypoxic chamber at 5,600 g for 4 minutes. The cell pellet was resuspended in 100 µl of TPGY broth prewarmed to about 37 °C. 100 µl aliquots of the broth containing the cells were plated onto TPGY agar containing 10 µg/ml erythromycin. The plates were incubated for four days.

C. *In vitro* Activity of Cytosine Deaminase from *C. Sporogenes*

Western blot analysis of cytosine deaminase protein expression in cell lysates of transformed *C. sporogenes* ("*C. sporogenes* w/pCD"), wild-type *C. sporogenes*, and *E. coli* transformed with the same vector ("*E. coli* w/pCD") was
5   performed. The results are shown in Fig. 10.

Conversion of nontoxic 5-fluorocytosine to toxic 5-fluorouracil by the cytosine deaminase-transformed *C. sporogenes* was evaluated by monitoring the growth inhibition of SCCVII cells incubated with 5-FC alone, 5-FU alone, or the addition of cell extract from recombinant *C. sporogenes* added to the culture. The
10   results are shown in Fig. 11.

D. *In vivo* Expression of Cytosine Deaminase and Antitumor Activity

The expression and activity of cytosine deaminase was evaluated in SCCVII tumor-bearing mice as follows. Tumor-bearing animals were injected
15   intraveneously (iv) with 10^8 spores of recombinant *C. sporogenes*, and 7 and 14 days later the tumors were excised and samples prepared for immunoblot analysis of cytosine deaminase and for cytosine deaminase activity *in vitro* as judged by the ability to convert 5-fluorocytosine to a more toxic product. The results are shown in Fig. 12.

20   In another study, SCCVII tumor-bearing mice were injected iv with 10^8 spores of recombinant *C. sporogenes* when the tumors were approximately 100 mg in size. Seven days after injection, the tumor, brain, heart, lung, liver, kidney, and spleen tissues were removed for immunoblot analysis. 100µg of cell extract from each of the tissues was loaded on gels and subjected to immunoblot analysis
25   following gel electrophoresis. As controls, 1 µg and 20 µg of cell extracts from cytosine deaminase-transformed *E. coli* and *C. sporogenes,* respectively, were loaded onto the gel. he results are shown in Fig. 12.

In another study, the ability of recombinant CD-expressing *C. sporogenes* to produce sufficient conversion of 5-FC to 5-FU *in vivo* to produce antitumor activity
30   was also evaluated. Groups of tumor-bearing mice with an average tumor size of 150 mg were injected with saline, 5-FC (500 mg/kg 5 times per week for 2 weeks), a maximum tolerated dose of 5-FU alone (16.6 mg/kg daily 5 days per week for 2 weeks), recombinant spores alone on day 0, or recombinant spores one day prior to daily injections of 5-FC. The results are shown in Fig. 14.

EXAMPLE 3

Expression of the β-glucuronidase gene (*uidA*) in *C. acetobutylicum*

Oligonucleotide primers, based on the published sequence, were utilized to amplify a fragment carrying the β-glucuronidase gene from *E. coli* K12 (Jefferson

5   *et al.*, 1986). One of the PCR primers employed created an *NdeI* site 'over' the *uidA* start codon, to facilitate insertion of the gene into pMTL500F, described in Example 1 and shown in Fig. 4, resulting in a modified form of the pMTL500F vector comprising the coding sequence for β-glucuronidase.

β-glucuronidase has to be exported out of the clostridial cell in order to be

10   active. To achieve this, the *uidA* gene was constructed with a 5' signal sequence to promote secretion of the translated protein by the classical signal peptide route (Bosslet *et al.*, 1992). Two signal sequences were employed: (1) the *Clostridium thermocellum celA* gene (Beguin *et al.*, 1985); and (2) the staphylococcal protein A gene (Shuttleworth *et al.*, 1987). The 5'-end of the *lacZ'* gene of pMTL500F (that

15   residing between the *NdeI* site and polylinker region) was replaced with DNA specifying the two signal sequences. The resulting plasmid was transformed into *C. acetobutylicum* NCIMB 8052 by electroporation.

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IT IS CLAIMED:

1. A composition comprising an obligately anaerobic bacteria of the genus *Clostridium* transformed with a recombinant expression vector including the coding sequence for an enzyme capable of catalyzing the conversion of a prodrug into a chemotherapeutic agent, wherein said bacteria is transformed under conditions effective to achieve a high level proliferation in a solid tumor *in vivo*.

2. The composition according to claim 1, wherein said conditions effective to achieve a high level proliferation include at least one of (i) presence of an inhibitor of nuclease activity; (ii) a temperature of less than about 10 °C and above freezing; or (iii) an environment having an oxygen level that maintains growth of said anaerobe.

3. The composition according to claim 2, wherein said conditions effective to achieve a high level proliferation include at least two of (i) presence of an inhibitor of nuclease activity; (ii) a temperature of less than about 10 °C and above freezing; or (iii) an environment having an oxygen level that maintains growth of said anaerobe.

4. The composition according to claim 2, wherein said conditions effective to achieve a high level proliferation include (i) presence of an inhibitor of nuclease activity; (ii) a temperature of less than about 10 °C and above freezing; and (iii) an environment having an oxygen level that maintains growth of said anaerobe.

5. The composition according to claim any one of the preceding claims, wherein the bacteria is *Clostridium sporogenes*.

6. The composition according to any one of claims 1-4, wherein the enzyme is selected from cytosine deaminase, nitroreductase, β-glucuronidase, β-galactosidase, carboxypeptidase, alkaline phosphatase, and β-lactamase.

7. The composition according to any one of claims 1-4, wherein the prodrug is selected from a glucuronide conjugate of epirubicin, a glucuronide conjugate of

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doxorubicin, 5-fluorocytosine, CB1954, 4-hydroxycyclophosphamide, hydroxyanaline mustard, etoposide phosphate, doxorubicin phosphate, mitomycin C phosphate, and 5-fluorocytosine.

5   8.   The composition according to any one of claims 1-4, wherein the prodrug is 5-fluorocytosine.

           9.   The composition according to any one of claims 1-4, wherein the enzyme is cytosine deaminase.

10

           10.   An obligately anaerobic *Clostridium* bacterial composition, comprising a coding sequence for an enzyme capable of catalyzing the conversion of a prodrug into a toxic agent, wherein said coding sequence is introduced into said bacteria under at least one of the following conditions: (i) presence of an inhibitor of

15   nuclease activity; (ii) a temperature of less than about 10 °C and above freezing; or (iii) an environment having an oxygen level that maintains growth of said anaerobe.

           11.   The obligately anaerobic bacterial composition according to claim 10,

20   wherein the conditions include (i), (ii) and (iii).

           12.   The obligately anaerobic bacterial composition according to claim 10 or claim 11, wherein the bacteria is *Clostridium sporogenes*.

25   13.   The obligately anaerobic bacterial composition according to any one of claims 10-12, wherein the enzyme is selected from cytosine deaminase, nitroreductase, β-glucuronidase, β-galactosidase, carboxypeptidase, alkaline phosphatase and β-lactamase.

30   14.   The anaerobic bacterial composition according to any one of claims 10-13, wherein the prodrug is selected from a glucuronide conjugate of epirubicin, a glucuronide conjugate of doxorubicin, 5-fluorocytosine, CB1954, 4-hydroxycyclophosphamide, hydroxyanaline mustard, etoposide phosphate, doxorubicin phosphate, mitomycin C phosphate, and 5-fluorocytosine.

15. The anaerobic bacterial composition according to claim 12, wherein the prodrug is 5-fluorocytosine and the enzyme is cytosine deaminase.

16. A composition for use in treating a tumor *in vivo* comprising the
5   bacterial composition according to any one of claims 1-15.

17. A method of transforming an anaerobe with a recombinant expression vector, comprising
contacting said anaerobe with a vector (i) in the presence of an inhibitor of
10   nuclease activity; (ii) at a temperature of less than about 10 °C and above freezing; and (iii) in an environment having an oxygen level that maintains growth of said anaerobe.

18. The method according to claim 17, wherein said contacting is at a
15   temperature of less than about 5 °C but above freezing.

19. The method according to claim 17 or claim 18, wherein said contacting is in the presence of a DNAase inhibitor.

20   20. The method according to any one of claims 17-19, further including the step of electroporating the anaerobe in the presence of the vector.

21. The method according to any one of claims 17-20, wherein said anaerobe is of the genus *Clostridia*.
25
22. The method of claim 22, wherein said *Clostridia* is *Clostridia sporogenes*.

23. A transformed bacteria prepared according to the method of any one of
30   claims 17-20.   .

24. The transformed bacteria according to claim 23 for use in treating a tumor in a subject.

45
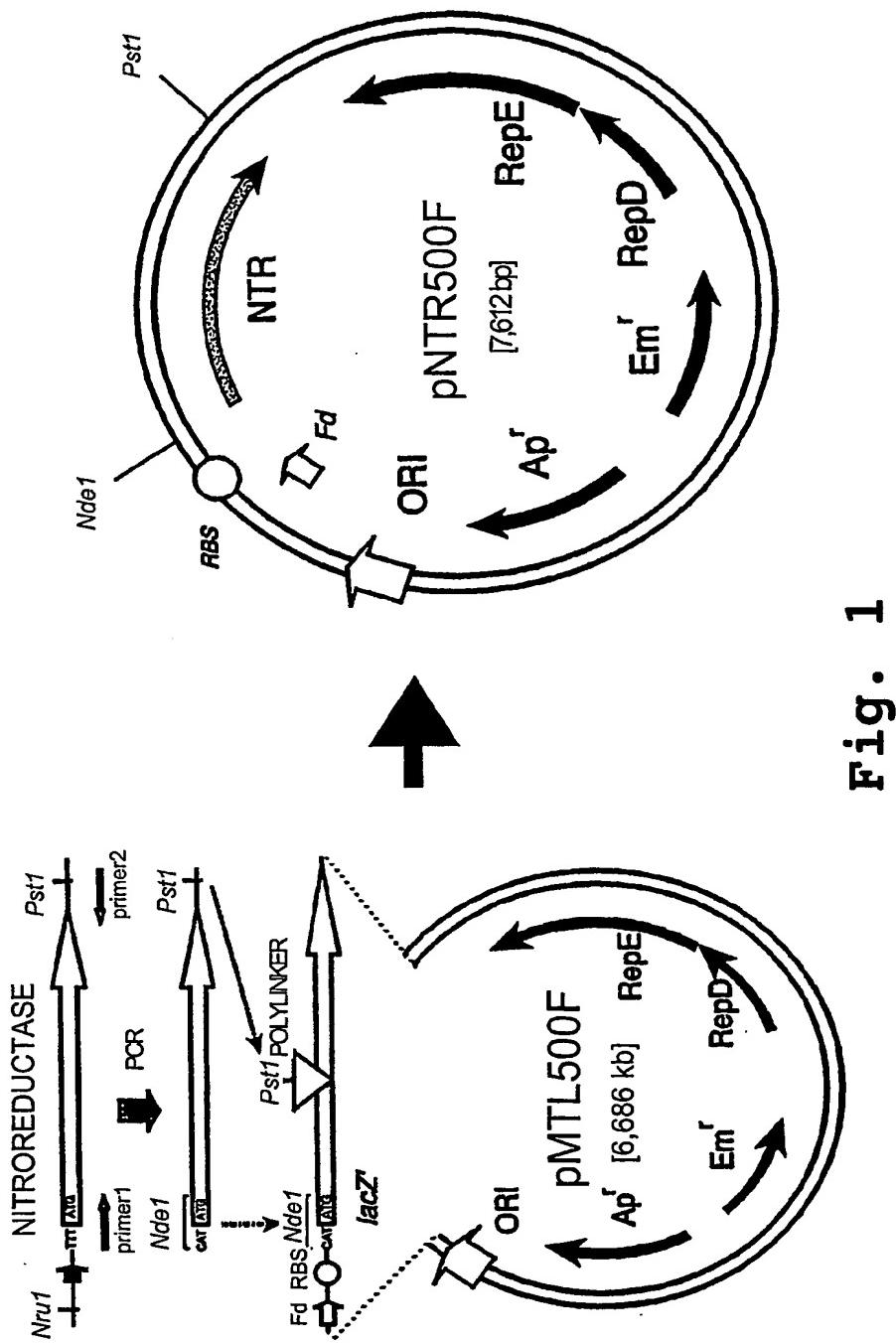
Fig. 1

```
                    MboI
                   ┌──────┐
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EcoRI

AAAGTTATAG AAGCAATAGA AGATTTAGGA TTTACTGTAA TATAAATTAC   100
     -35          promoter        -10                 ┌──────────
  ───────                      ──────────          ┌
ACTTTTAAAA AGTTTAAAAA CATGATACAA TAAGTTATGG TtGGAATTGT   150
    lac operator                    ribosome binding site
  ─────────────────────┐          ┌─────────────────┐
TATCCGCTCA CAATTCCAAC TTATGATTAA AATTTTAAGGA GGTGTATTT   200
  ─────────────────────┘          └─────────────────┘

cat  ┌─────┐  ◄──── START CODON lacZ'
     │ ATG │
     └─────┘
   NdeI
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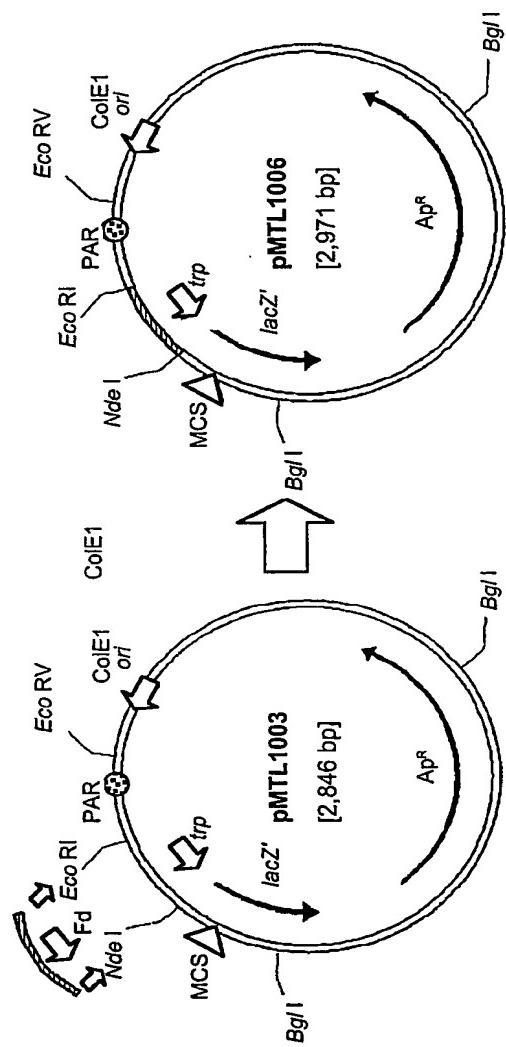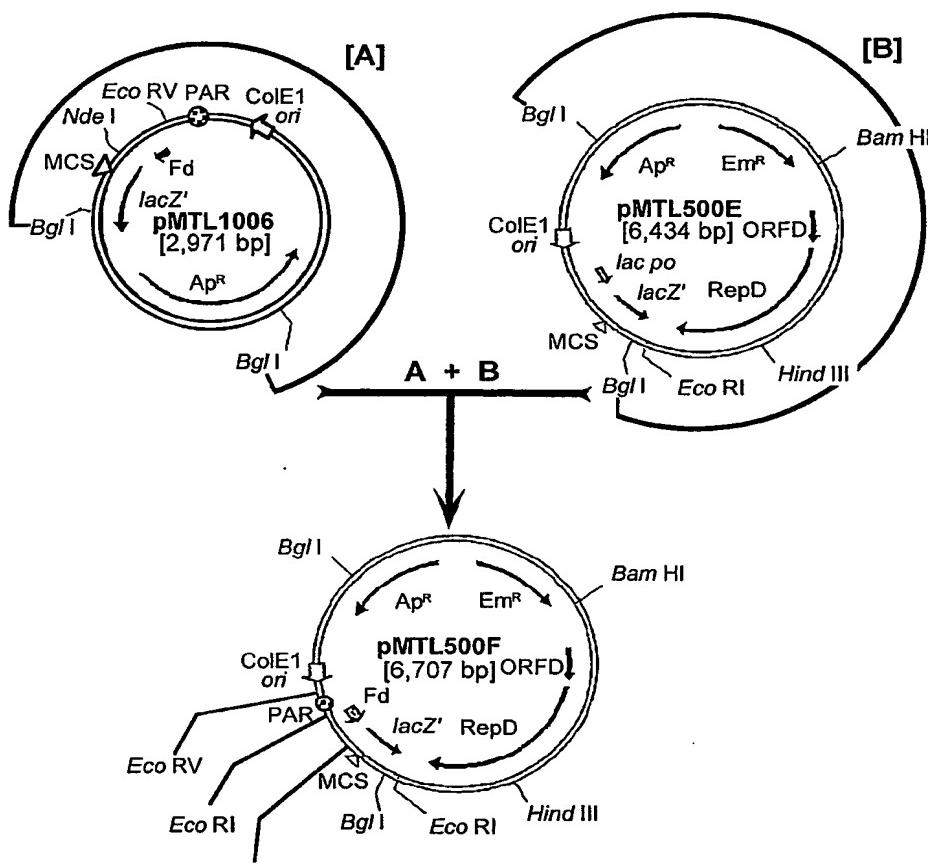
Fig. 2

Fig. 3

Fig. 4
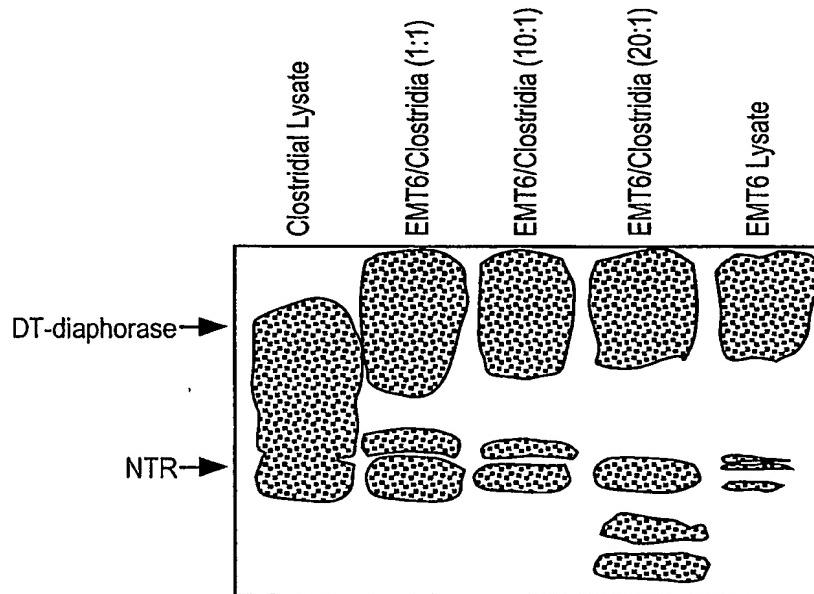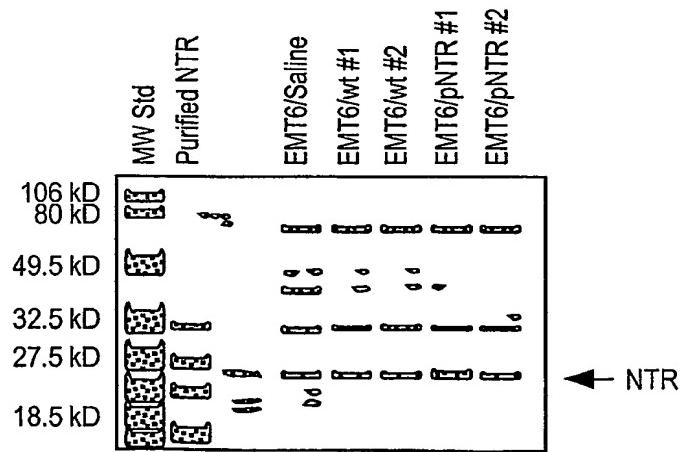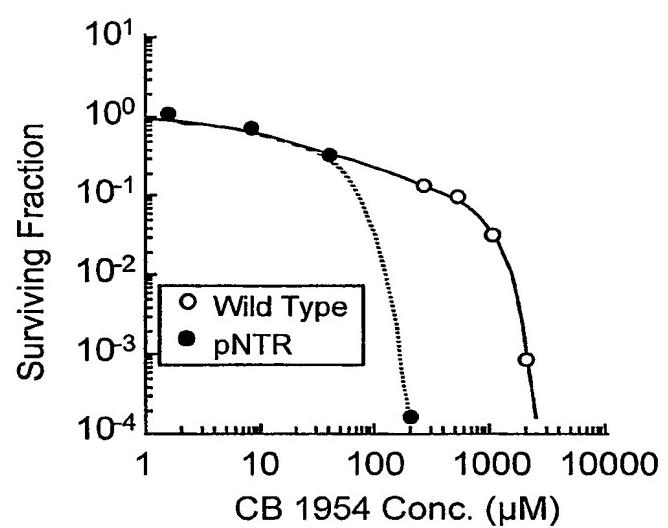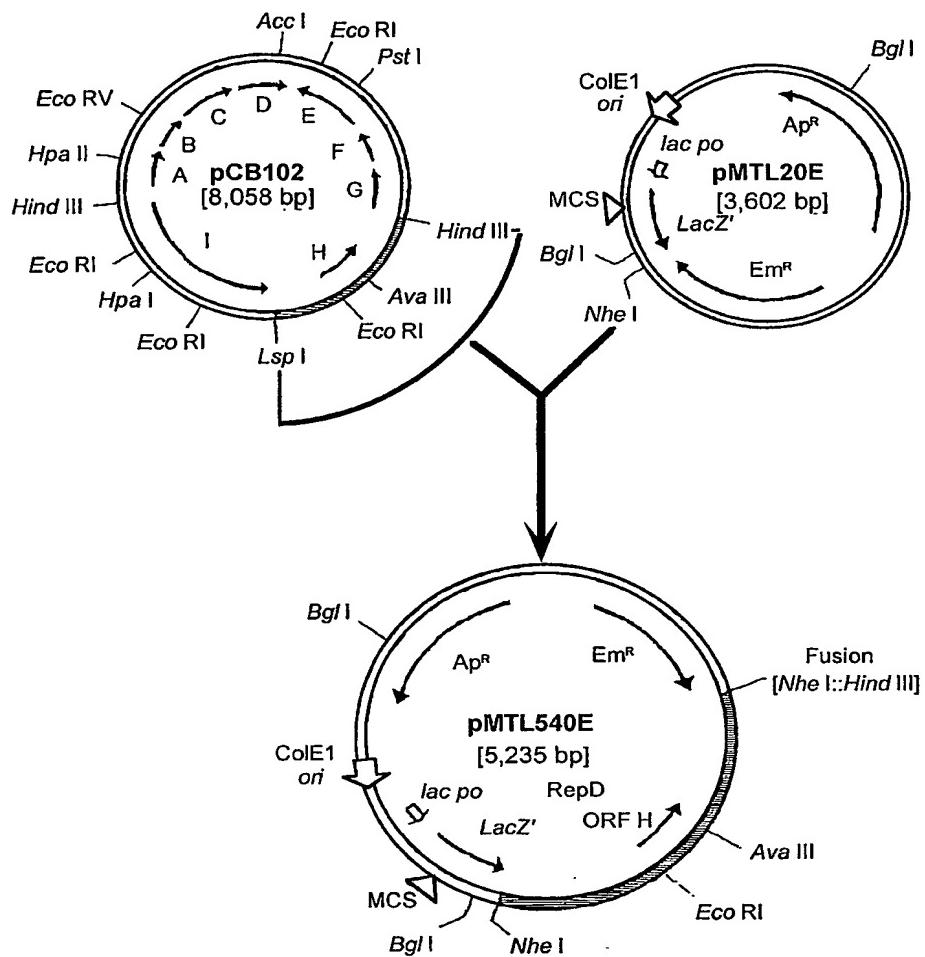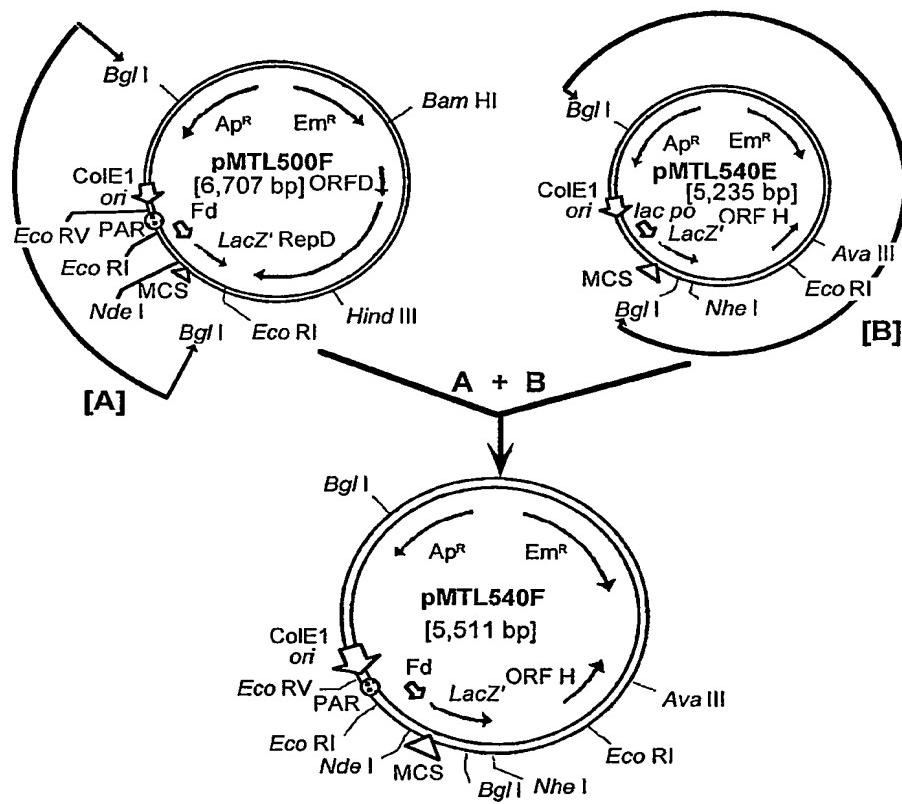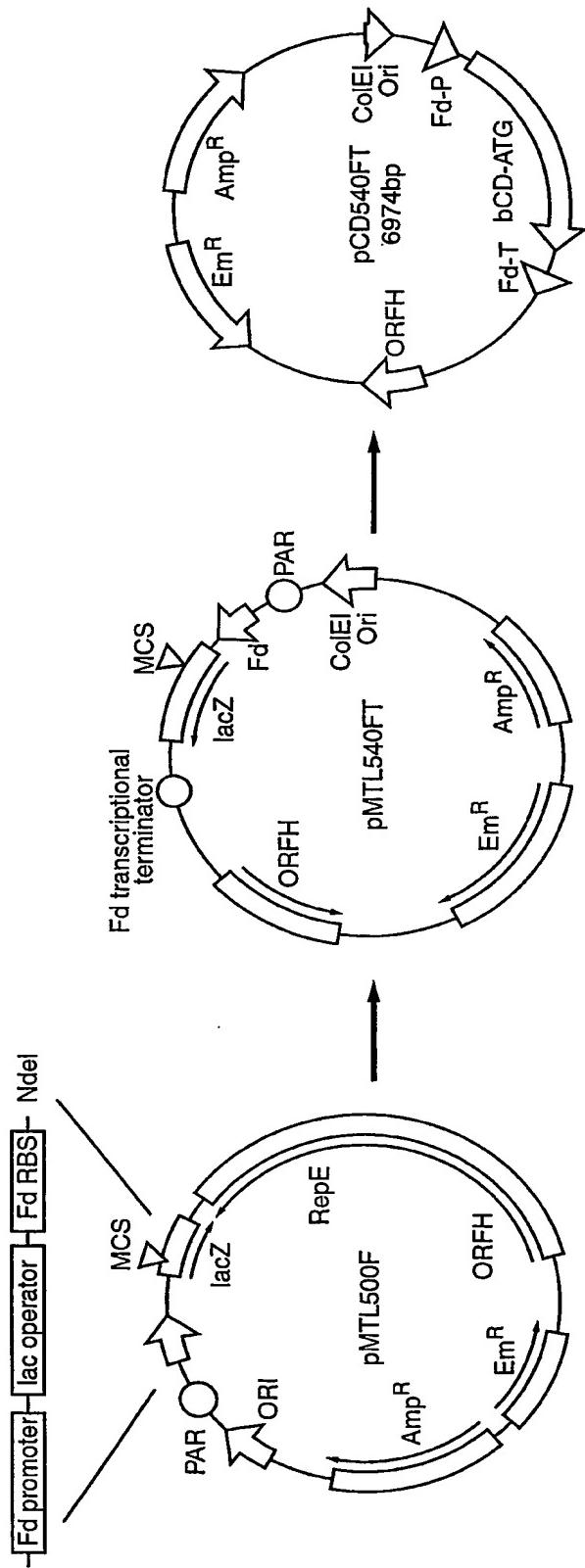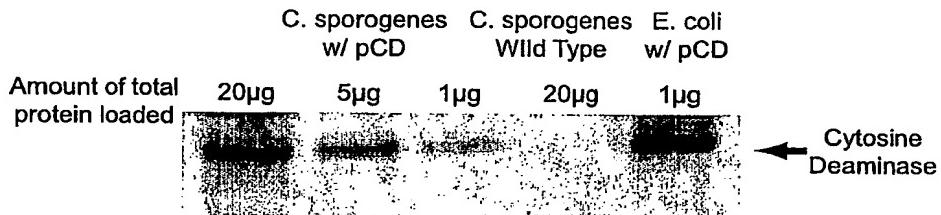
Fig. 5A

Fig. 5B

Fig. 6

Fig. 7

Fig. 8

Fig. 9

C. sporogenes   C. sporogenes   E. coli
w/ pCD   Wild Type   w/ pCD

Amount of total   20µg   5µg   1µg   20µg   1µg
protein loaded   ← Cytosine
                                                                          Deaminase

Fig. 10

○ 5-FC only
◆ 5-FC+CE (5 µg/ml)
▼ 5-FC+CE (10 µg/ml)   ● 5-FU only
▲ 5-FC+CE (20 µg/ml)   ● 5-FU+CE (20 µg/ml)
                          □ 5-FC + CE from empty vector
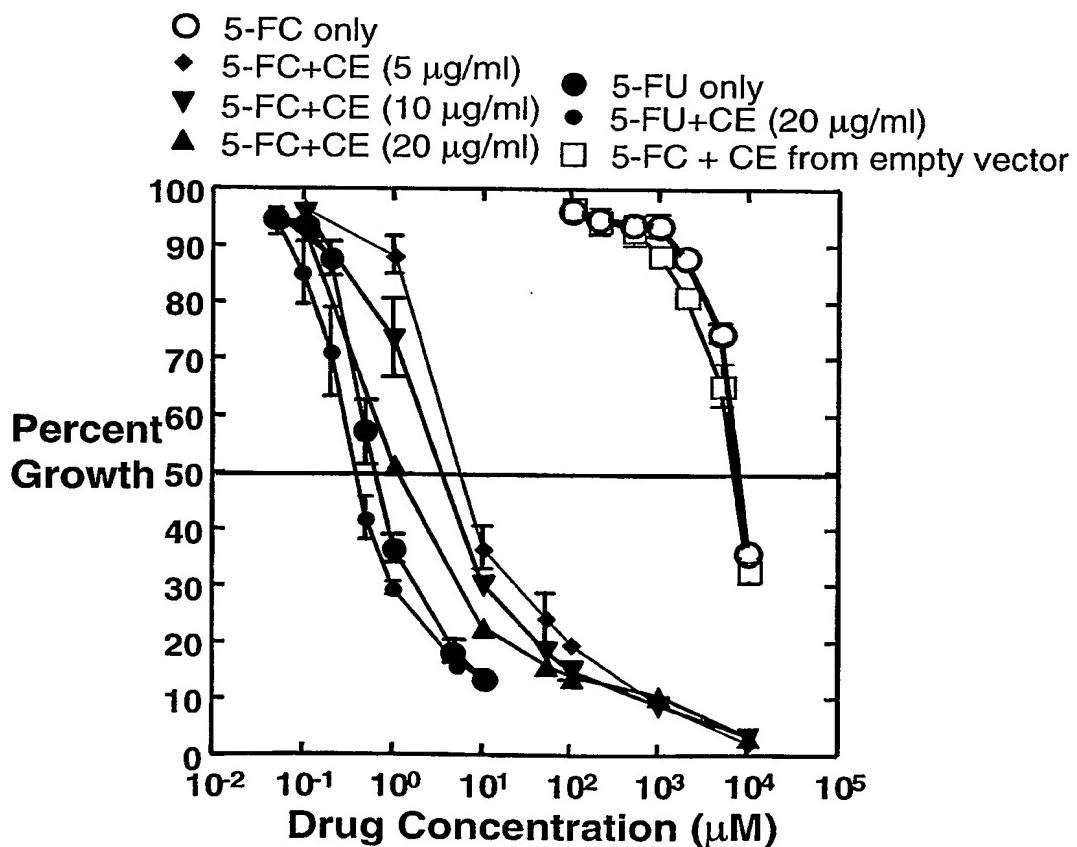
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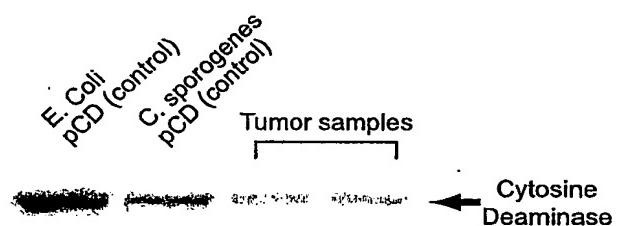
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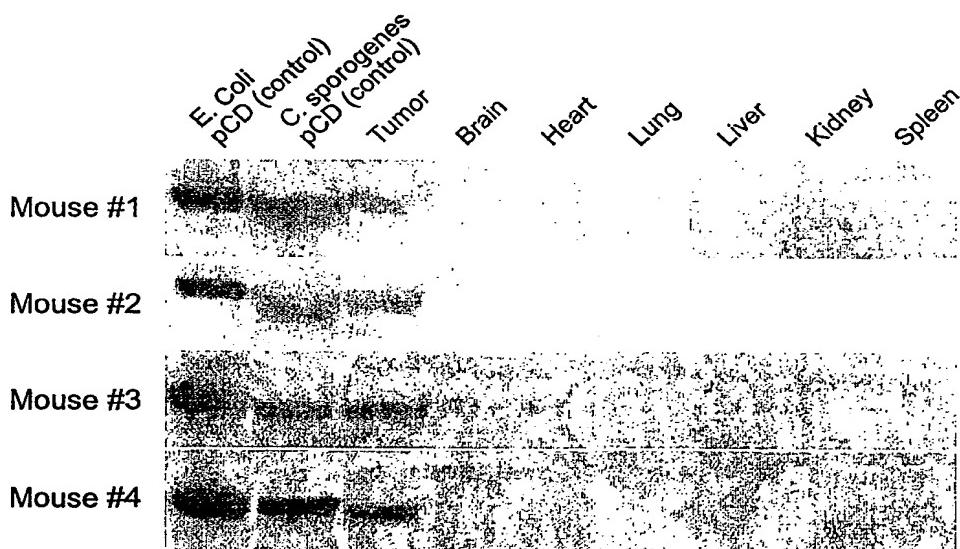
Fig. 13
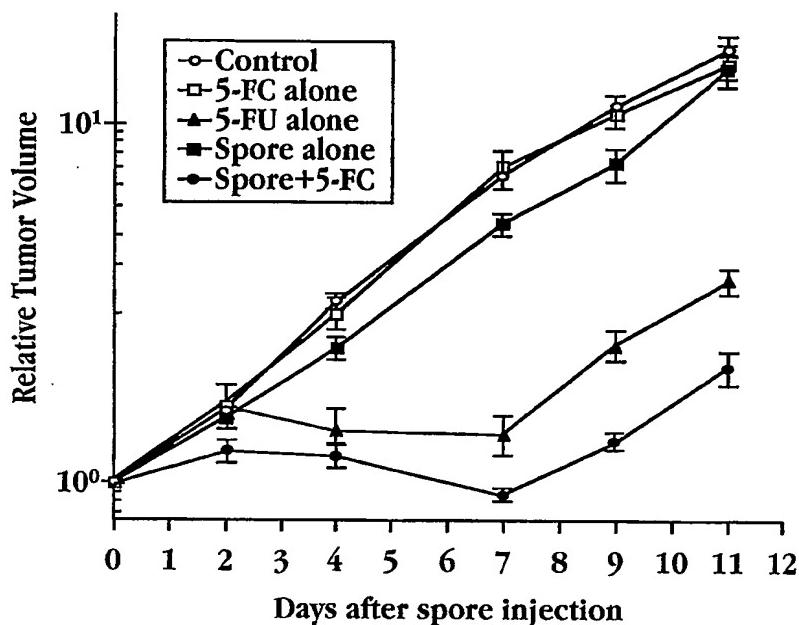
Fig. 14
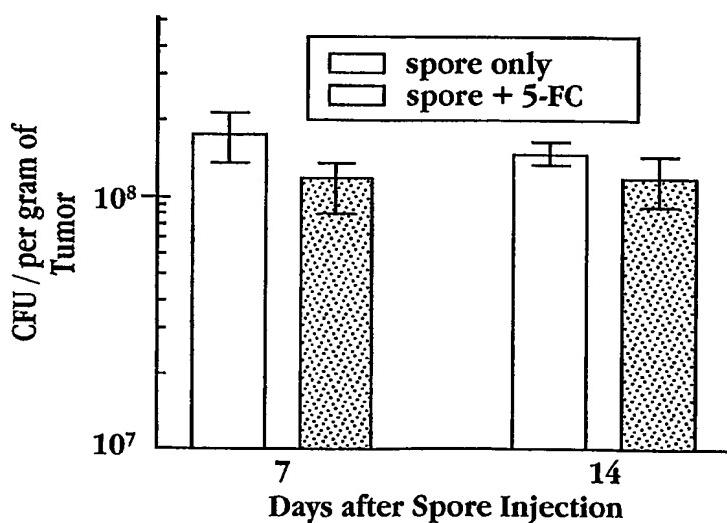
Fig. 15

SEQUENCE LISTING

<110> The Board of Trustees of the Leland
   Stanford Junior University
       Centre for Applied Microbiology &
   Research

<120> Anaerobe Targeted Enzyme Mediated
   Prodrug Therapy

<130> 586008169W00

<140> Not Yet Assigned
<141> Filed Herewith

<150> US 10/158,976
<151> 2002-05-31

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```

1

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 1/20, 15/74
US CL : 435/252.3, 252.7, 471, 479, 480
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
    U.S. : 435/252.3, 252.7, 471, 479, 480

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEMMMON, M.J. et al. Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. Gene Therapy. 1997 Vol. 4, pages 791-796, entire document.	1-12, 19
A	NUYTS, S. et al. Radio-responsive recA promoter significantly increases TNA-alpha production in recombinant clostridia after 2 Gy irradiation. Gene Therapy. 2001 Vol. 8 pages 1197-1201.	1-5, 10-12, 19

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier application or patent published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 August 2003 (08.08.2003)	30 OCT 2003
Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US	Konstantina Katcheves
Commissioner for Patents	
P.O. Box 1450	
Alexandria, Virginia 22313-1450	
Facsimile No. (703)305-3230	Telephone No. (703) 305-3388

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/17291

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 13-14, 16 and 20-24
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
                        ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
EAST, STN, MEDLINE
search terms: clostridium, gene therapy, cancer, tumor, vector constructs, transformation

(54) Title: ANAEROBE TARGETED ENZYME-MEDIATED PRODRUG THERAPY

(57) Abstract: Recombinant anaerobic bacterial compositions that under anaerobic conditions present in a solid tumor and produce an enzyme capable of catalyzing the conversion of a prodrug to its cytotoxic product *in situ* are described. Methods of treating tumors using the composition are also described.

AMENDED CLAIMS

received by the International Bureau on 02 December 2003 (02.12..2003)
original claims 13, 14, 16, 20-23, replaced by claims bearing the same number.

doxorubicin, 5-fluorocytosine, CB1954, 4-hydroxycyclophosphamide, hydroxyanaline mustard, etoposide phosphate, doxorubicin phosphate, mitomycin C phosphate, and 5-fluorocytosine.

8. The composition according to any one of claims 1-4, wherein the prodrug is 5-fluorocytosine.

9. The composition according to any one of claims 1-4, wherein the enzyme is cytosine deaminase.

10. An obligately anaerobic *Clostridium* bacterial composition, comprising a coding sequence for an enzyme capable of catalyzing the conversion of a prodrug into a toxic agent, wherein said coding sequence is introduced into said bacteria under at least one of the following conditions: (i) presence of an inhibitor of nuclease activity; (ii) a temperature of less than about 10 °C and above freezing; or (iii) an environment having an oxygen level that maintains growth of said anaerobe.

11. The obligately anaerobic bacterial composition according to claim 10, wherein the conditions include (i), (ii) and (iii).

12. The obligately anaerobic bacterial composition according to claim 10 or claim 11, wherein the bacteria is *Clostridium sporogenes*.

13. The obligately anaerobic bacterial composition according to claim 10 or claim 11, wherein the enzyme is selected from cytosine deaminase, . nitroreductase, β-glucuronidase, β-galactosidase, carboxypeptidase, alkaline phosphatase and β-lactamase.

14. The anaerobic bacterial composition according to claim 10 or claim 11, wherein the prodrug is selected from a glucuronide conjugate of epirubicin, a glucuronide conjugate of doxorubicin, 5-fluorocytosine, CB1954, 4-hydroxycyclophosphamide, hydroxyanaline mustard, etoposide phosphate, doxorubicin phosphate, mitomycin C phosphate, and 5-fluorocytosine.

AMENDED SHEET (ARTICLE 19)

15. The anaerobic bacterial composition according to claim 12, wherein the prodrug is 5-fluorocytosine and the enzyme is cytosine deaminase.

16. A composition for use in treating a tumor *in vivo* comprising the bacterial composition according to any one of claims 1-4, 10, or 11.

17. A method of transforming an anaerobe with a recombinant expression vector, comprising

contacting said anaerobe with a vector (i) in the presence of an inhibitor of nuclease activity; (ii) at a temperature of less than about 10 °C and above freezing; and (iii) in an environment having an oxygen level that maintains growth of said anaerobe.

18. The method according to claim 17, wherein said contacting is at a temperature of less than about 5 °C but above freezing.

19. The method according to claim 17 or claim 18, wherein said contacting is in the presence of a DNAase inhibitor.

20. The method according to claim 17 or claim 18, further including the step of electroporating the anaerobe in the presence of the vector.

21. The method according to claim 17 or claim 18, wherein said anaerobe is of the genus *Clostridia*.

22. The method of claim 21, wherein said *Clostridia* is *Clostridia sporogenes*.

23. A transformed bacteria prepared according to the method of claim 17 or claim 18.

24. The transformed bacteria according to claim 23 for use in treating a tumor in a subject.

AMENDED SHEET (ARTICLE 19)

and a gate of each another NMOS transistor connected to the gate another one of the NMOS transistors.

6.   The circuit according to claim 5 further including an inductive load coupled to the differential output.

7.   The circuit according to claim 6 further including a MOS transistor switch coupled between the connected sources of the NMOS transistors and ground.

8.   The circuit according to claim 7 further including an inductor coupled between the connected sources of the NMOS transistors and the drain of the MOS transistor switch.

9.   The circuit according to claim 6 further including a current source coupled between the connected sources of the NMOS transistors and ground.

10.   The circuit according to claim 8·further including a switched variable capacitor coupled between the connected source of the NMOS transistors and ground.

11.   The circuit according to claim 5 wherein the differential pair of transistors does not contain additional transistors arranged in a cascode configuration.

12.   The circuit according to claim 5 wherein the differential pair of transistors is an RF differential pair of transistors.

13.   The circuit according to claim 2 further including an inductive load coupled to the differential output.

14.   The circuit according to claim 2 further including a MOS transistor switch coupled between the differential pair of transistors and ground.

15.   The circuit according to claim 14 further including an inductor coupled between the source of differential pair of transistors and the drain of the MOS transistor switch.

AMENDED SHEET (ARTICLE 19)